DEVELOPMENT AND USE OF ALTERNATIVE TECHNOLOGY BASED ON BIOLOGICAL AND BORON FERTILIZER TO ALLEVIATE COLD STRESS IN AGRICULTURE

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DEVELOPMENT AND USE OF ALTERNATIVE TECHNOLOGY BASED ON BIOLOGICAL AND BORON FERTILIZER TO ALLEVIATE COLD STRESS IN AGRICULTURE

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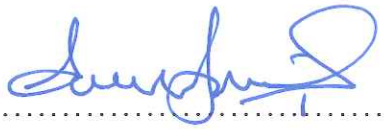
Assoc. Prof. Dr. Adem Güneş ..... 
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ABSTRACT

DEVELOPMENT AND USE OF ALTERNATIVE TECHNOLOGY BASED ON BIOLOGICAL AND BORON FERTILIZER TO ALLEVIATE COLD STRESS IN AGRICULTURE

Turkey's climatic conditions have shown higher temperature variations in recent years. Low temperature is one of the most important factors limiting crop production. A significant annual loss in crop yield and quality have been reported in each region. Recently, some organic and inorganic mineral resources are being used as frost resistance mechanisms to help plants overcome frost stress. In this study the effects of Bio-B formulation developed such as reduction of low temperature and frost damage, antifreeze protein, ice nucleation activity, antioxidant activity, hormone and amino due to acid balance were investigated on wheat, by evaluating biochemical variations revealed in the preliminary plant and soil analyzes in Erzurum in Turkey. In addition to biochemical tests of different microorganisms used in bio-B formulations, the ability to produce antifreeze proteins at low temperatures and their b were tested. *Azosprillum brasilence* bacterium was determined as the most effective organism. Biochemical analyzes of *Azosprillium brasilense* and combined B formulations were tested for pathogens such as *Pseudomonas syringae*. As a result of the test, the most suitable boron level was 0.5 percent and the most suitable *Azosprillium brasilense* content was 10^6-10^7 CFU / ml. The determined organism and B formulations were applied in an area in Erzurum for wheat plant. According to results, wheat yield increased by 12.88 percent compared to control with Bio-B application from soil. In other applications, wheat yield was increased by 4.68 percent from foliar increased by 8 percent soil + foliar application. With the soil application the highest yield and the highest amino acid and organic acid and the highest soil B content are obtained. The lowest frost damage rate was obtained by foliar Bio-B application. According to the control (no Bio-B fertilizer application), the frost damage rate decreased in case of Bio-B application from soil as 17.99 percent; as 23.15 percent with foliar application and as 21.54 percent with soil + foliar application.

ÖZET

BİYOLOJİK GÜBRE VE BORA DAYALI ALTERNATİF TEKNOLOJİ İLE TARIMDA DON ZARARINI AZALTAN GÜBRE FORMULASYONU GELİŞTİRİLMESİ

Türkiye'nin sahip olduğu coğrafik konum gereği, yıllık sıcaklık değişimleri oldukça yüksek varyasyonlar göstermektedir. Son zamanlarda don stresinin çözümüne yönelik bazı organik ve inorganik mineral kaynaklar kullanılarak, bitkilerin dona dayanım mekanizmaları artırılmaya çalışılmaktadır. Erzurumda yetiştiriciliği yapılan buğday bitkisinde, düşük sıcaklık ve don hasarının azaltılmasında, geliştirilen Bio-B formulasyonunun etkileri ortaya konmaya çalışılmıştır. Buğday bitkisinde Bio-B gübre uygulamasına bağlı olarak meydana gelen biokimyasal değişimler, verim ve kalite parametreleri de belirlenmiştir. Bio-B formulasyonunda kullanılan farklı mikroorganizmaların biyokimyasal testleri yanında düşük sıcaklıklardaki antifiriz protein üretme kabiliyetleri ve etkinlikleri testi yapılmıştır. Çalışma sonununca elde edilen *Azosprillum brasilence* bakterisi en etkin organizma olarak belirlendi. Belirlenen organizma ve oluşturulan B formülasyonu Erzurum ilinde buğdaya, toprak, yaprak ve toprak+yapraktan belirlenen dönemlerde uygulanmıştır. Buğday bitkisinde topraktan Bio-B uygulaması ile en yüksek toprak B içeriği, en yüksek buğday verimi, en yüksek amino asit ve organik asit miktarı elde edilmiştir. En düşük don hasarlanma oranı ise yapraktan Bio-B uygulaması ile elde edilmiştir. Buğday bitkisinde topraktan Bio-B uygulaması ile kontrole göre buğday verimi yüzde 12,88 oranında artış göstermiştir. Diğer uygulamalarda ise buğday verimi kontrole göre, yapraktan uygulama ile yüzde 4,68; toprak+yaprak uygulaması ile yüzde 8 oranında artış göstermiştir. Donma hasar oranında ise hiç bir Bio-B gübre uygulaması yapılmayan kontrole göre topraktan Bio-B uygulaması durumunda hasar oranı yüzde 17,99; yapraktan uygulama ile yüzde 23,15 ve toprak+yapraktan uygulama ile yüzde 21,54 oranlarında azalma göstermiştir. *Azosprillium brasilense* ait biyokimyasal analizler yapılmıştır. Bu test sonucunda en uygun B düzeyi yüzde 0,5 ve en uygun *Azosprillium brasilense* miktarının ise 10^6 - 10^7 cfu/ml olduğu belirlenmiştir.

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LIST OF SYMBOLS/ ABBREVIATIONS

ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
AFP	Antifreeze proteins
CAT	Catalase
COR	Cold regulated protein
CS	Cold stress
DHN	Dehydrin protein
Fe_2O_3	Iron oxide
GA_3	Gibberellic acid
H_2O_2	Hydrogen peroxide
HSP	Heat shock protein
IAA	Indole-3-asetic acid
INA	Ice nucleating active bacteria
OH^-	Hydroxyl radical
PGPR	Plant growth promoting rhizobacteria
POD	Peroxidase
ROS	Reactive oxygen species
SOD	Superoxide dismutase
UV	Ultraviolet

1. INTRODUCTION

1.1. DEFINITION OF COLD STRESS

Global warming and abnormal climate change which is seen in past years and expected to continue drastically, causes unstable climate conditions and increases natural disasters both in number and in grade. Due to changes in climate, biotic and abiotic stress has been becoming an important issue in the agriculture [1]. Environmental conditions like drought, high soil salinity\sodicity, acidic or alkaline soils, low and high temperature, heavy metals, exposure to more UV radiation, floods and lack of nutrients are considered abiotic stress affecting plant growth and productivity adversely [2]. Biotic stress, on the other hand, is referred to a damage caused by living organisms such as bacteria, viruses, fungus, nematodes and insects [3].

Among various stresses, low temperature (cold) is one of the most harmful factors constraining the agricultural productivity and spatial distribution of many species, including important agricultural crops [4]. According to Food and Agriculture Organization, in USA, freezing of crops have caused more economic losses than any other weather parameters [5].

Cold stress (CS) occurs when plants are exposed to chilling (0–20 °C) and freezing temperature (< 0 °C). Plant response to low temperature shows a great variability according to its structure, physiological and genetic factors as well as geographical region and environmental conditions. Chilling sensitive plants such as tropical and subtropical plant species cannot tolerate chilling temperatures (0–20 °C) whereas chilling resistant plants from temperate regions can tolerate subzero temperatures. Freezing sensitive plants on the other hand, cannot tolerate subzero temperature while many temperate woody conifer and deciduous plant species could survive extreme low temperatures even below −196 °C which are named as freezing tolerant plants [6].

Resistance to stress conditions in plants is provided in two ways. These are;

i. Avoidance (reducing or blocking the existing stress factor),

ii. Tolerance (survive against and stress with no or slight damage).

1.2. FREEZING OF PLANTS

Freezing stress affects cells much more drastically than the chilling stress because of the formation of ice crystals in the intracellular spaces leading to irreversible cell death. Freezing injury in fact starts when ice is formed in the apoplastic (extracellular) spaces of plant tissues.

As temperature decreases slightly slow to a subzero temperature, ice formation occurs first in the xylem and vascular elements and grows in the apoplastic spaces thereafter because apoplastic space has relatively lower solute concentration. At any given temperature, vapor pressure of ice is much lower than the cell water. Vapor pressure gradient establishes between extracellular ice crystals and (cytoplasmic) intracellular liquid water. This gradient enables intracellular water migrates down to extracellular space which adds existing ice crystals in the extracellular space. Freezing point (melting point) of cellular water in the cell is decreases as the water moves out. Therefore, ice formation in the cell is prevented to some extent. Extracellular freezing causes cell shrinkage, dehydration and concentration of solutes in plant tissues which can be tolerated to varying degrees. If the rate of cooling is sufficiently low, the cell water continues to migrate down until the equilibrium between the intra- and extracellular water is reached. Intracellular ice will not occur and thus, no lethal injury is observed [7, 8].

1.3. PLANT SUPERCOOLING

Very sparsely, tissue water remains liquid below at its actual freezing point (melting point) but rather supercools to varying degrees before it freezes. This is a metastable state of water and is destroyed by ice nucleation. Degree of supercooling process is determined by the ice nucleators on the plant surface and its environment. Ice nucleators can be either intrinsic or extrinsic. Intrinsic ice nucleators are produced by plants. Extrinsic nucleators are produced by other source rather than the organisms itself such as *Pseudomonas*, *Xanthomonas* and *Erwinia*, or fungi such as *Fusarium*, which often present in the phyllosphere. Extrinsic nucleators are produced by ice nucleating active bacteria (INA). Species and sources of ice-nucleation active bacteria are shown in Table 1.1. [9].

Table 1.1. Species and sources of ice-nucleation active bacteria

Source	**Species**
Plant	
Leaves and other aerial parts of plants	*Pseudomonas syringe* *P. fluorescens* *P. viridiflava* *P. chlororaphis* *Erwinia herbicola* *E. ananas* *E. uredovora* *Xanthomonas campestris pv. transluces* *X. campestris*
Xylem of roots of alfafa (and adjacent soil)	*P. syringae, P. fluorescens, E. herbicola*

1.4. COLD ACCLIMATION

In order to deal with low temperature (0-15 °C), but not freezing temperatures, number of plants species are trying to continue homeostasis, induce a consecutive event that cause changes in gene expression so trigger biochemical modifications to increase their degree freezing tolerance in response. This phenomenon is called cold acclimation. Under exposure of low temperature, it is observed that there is a series of physiological, morphological, biochemical, metabolic and molecular changes occur within plants [11]. Basics of cold acclimation is as shown in Figure 1.1.

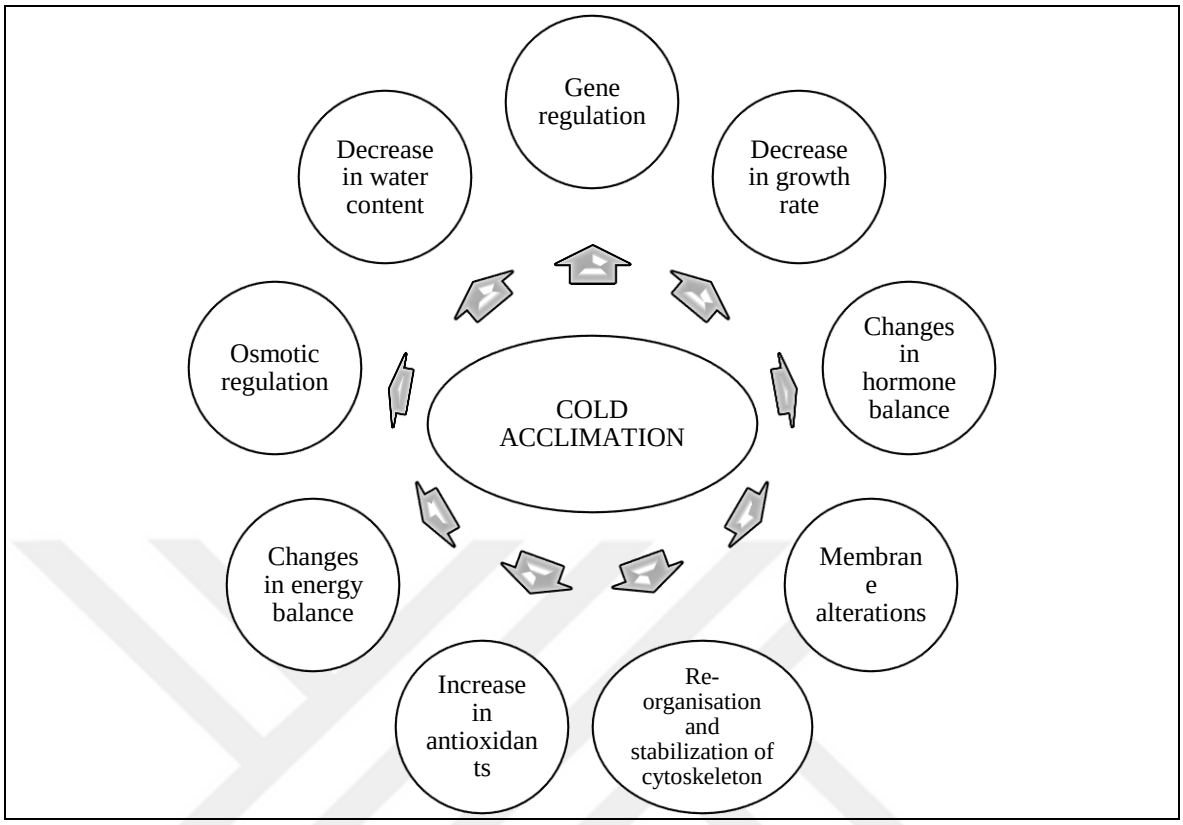


Figure 1.1. Basics of cold acclimation

1.4.1. Physiological Changes in Plants

The survival of plants in environmental extreme conditions depends on their ability to adapt morphologically and physiologically to these conditions. After effect of low temperature, various phenotypic changes are seen in plants such as yellowing of leaves (chlorosis), decreased foliar expansion, wilting, reduces tillering, withering, poor germination, stunted seedlings or at the end maybe death of tissue (necrosis) [10, 11].

The magnitude of damage is high when cold stress occurs at plant's green parts and reproductive parts during reproductive stage [12]. Plant heading was delayed in the course of exposure of low temperature and it causes pollen sterility which results in serious yield loss in agriculture [13].

1.4.2. Biochemical Changes in Plant Cell

Biochemical changes that occur during cold acclimation such as changes in membrane lipid composition, changes of soluble substances in plant sap, amount of protein, enzyme activity, change in antioxidant system and changes in plant nutrient elements are very important in low temperature resistance mechanisms of plants. The effects of these molecules are handled separately [14].

1.4.2.1.Membrane Lipid Composition

The primary adverse effect of low temperature is that it brings about serious plasma membrane damage by changing in its composition, structure, and function. Exposure of low temperature decreases fluidity of cell membrane and increases rigidity leading to solute leakage. It is mostly linked to the cold stress-induced dehydration [15]. Plasma membrane is composed of lipids and proteins. There are two kinds of lipids found in plasma membrane, i.e. saturated and unsaturated fatty acids. Fluidity and functionality of membrane depends on the composition of saturated and unsaturated fatty acids in the plant cell membrane [15]. Increasing saturated fatty acid leads to increase solidification in the cell membrane during cold stress.

1.4.2.2. Soluble Substances in Plant

Another typical changes during cold acclimation is synthesis of cryoprotectants which have an important role protecting cell membrane and organelles from freezing damage. Cryoprotectant molecules such as sugar alcohols (sorbitol, inositol, ribitol) soluble sugars (saccharose, starchyose, raffinose, trehalose) and low-molecular weight nitrogenous compounds (proline, glycine, betaine) provide osmatic regulations for perpetual water intake by cooperating dehydrin proteins (DHNs), cold regulated proteins (CORs), and heat shock proteins (HSPs). In conjunction, they try to bring into a balance membrane phospholipids and proteins, sustain ion continuity, and scavenging reactive oxygen species (ROS).

Some of the specific proteins that accumulate during the cold acclimation show anti-freeze protein properties [16]. Antifreeze proteins (AFPs) have the ability to alter the shape and formation of the ice crystals and prevent the recrystallization of ice. As a result, they contribute to the protection of cells from frost damage. During the cold acclimation of barley, wheat, rye and winter canola, AFP accumulation was observed [17, 18]. These proteins, which have the property of detergents, are non-water repellent and preventive of macromolecular aggregation [19, 20]. Cold acclimation and changing gene expression were determined in some studies. Many studies have shown that new polypeptides are synthesized in response to low temperatures. In recent years, studies have shown that there is a connection between frost resistance and proteins. It was determined that the beginning of cold acclimation of these polypeptides was related to frost resistance. Their role in frost resistance is increased at the beginning of the resistance process and eliminated in the deaclimation process.

1.4.2.3 Antioxidant System and Enzyme Activity

Cold stress induces oxidative stress in plants by accumulation of these reactive oxygen species which are partially reduced forms of atmospheric oxygen, highly reactive, toxic and led to damage cell structures, carbohydrates, lipids, proteins and DNA [16]. Singlet oxygen ($^1O_2$), superoxide ($^{\bullet}O^{-}_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) are common reactive oxygen species. To prevent cell against these adverse effects of reactive oxygen species, antioxidant enzymes are produced by plants. Superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) are significant antioxidant enzymes increased in cold stress. Superoxide dismutase (SOD) is a scavenger of $^{\bullet}O^{-}_2$ and hydrogen peroxide is produced after enzymatic reaction. Catalase (CAT) scavenges hydrogen peroxide. The reactions are as follows [21];

$$O_2 + e^- \rightarrow {}^{\bullet}O^{-}_2 \quad (1.1)$$

$$2\,H^+ + {}^{\bullet}O^{-}_2 + {}^{\bullet}O^{-}_2 \rightarrow H_2O_2 + O_2 \quad (1.2)$$

$$H_2O_2 + e^- \rightarrow HO^- + {}^{\bullet}OH \quad (1.3)$$

$$2\,H^+ + 2\,e^- + H_2O_2 \rightarrow 2\,H_2O \quad (1.4)$$

Peroxidase (POD) is also scevengers of hyrogen peroxide and converts it to water.

$$RH_2 + H_2O_2 \rightarrow 2\,H_2O + R \quad (1.5)$$

Peroxidase participates in lignification and indole 3-acetic acid degradation.

1.5. COLD STRESS TOLERANCE OF PLANTS IN TURKEY

Ertürk and Güleryüz [22] conducted a study on frost resistance of some local and foreign apricot varieties in Erzincan conditions, in November (beginning of rest), January (mid-rest) and March (end of rest). According to the results of artificial frost tests performed at different times to the buds collected in the specified months, it was found that the degree of durability varied between varieties in terms of periods and years. In the frost test, the rate of damage and the electrical conductivity and conductivity of the buds were found to be positively correlated, whereas the water content of the buds was negatively correlated. The total amount of sugar, protein and lipid content in the buds of apricot varieties increased from the beginning to the end of the rest and the starch content was decreased.

In another study conducted in Erzincan ecology, some varieties of native and foreign originated almond had showed different cold stress patterns. According to the artificial frost tests late-awakening genotypes were found more advantageous. It was determined that the flower bud's frost resistance was positively correlated with dry matter and Ca content, and negatively correlated with Cu content [23].

Another study involves changes in the metabolic pathways against cold stress of *Arabidopsis thaliana* plant, which can adapt to almost all parts of the world. It has been shown that energy mobilization with glycolysis occurs as a result of the cold stress created in Gebze University laboratory conditions, and ethanol degradation has occurred in order to increase TCA activity through acetyl-CoA [24].

The effect of salicylic acid was evaluated in two species of Hordeum vulgare culture plant which are cold sensitive and cold tolerant. Accordingly, after applying salicylic acid at 20-18 ° C, the seeds were stored at 7-5 ° C. After three days, exogenous salicylic acid was found to increase cold tolerance by regulating the activities of apoplastic antioxidative enzyme, ice nucleation and apoplastic proteins [25].

Koc conducted a study evaluating gene regulations against cold stress in *Nicotiana benthamiana* crop from *Solanaceae* family. As a result, stress, lipid, carbohydrate,

signaling associated gene expressions were found to be upregulated such as bHLH, E2F/DP, bZIP, SET, Homeobox, GRAS, ARF [26].

1.6. PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

1.6.1. History of Plant Growth Promoting Rhizobacteria

Inoculation of plants with beneficial bacteria has been continuing for centuries. Farmers have experienced the increase in crops when they cultivate in a leguminous soil. At the end of the 19th century, the application of mixing the soil with seeds which is naturally inoculated with bacteria, has emerged as a method in the US and became widespread. Ten years later, the first patent has been registered as *Rhizobium sp* (Nitragin'). After the registration, inoculation of legumes with rhizobia became widespread. Retrospectively, although more than 100 years of small-scale companies engaged in seed inoculation with *Rhizobium* have been established, significant contributions have been made in Australia, North America, Eastern Europe, Egypt, Israel, South Africa, New Zealand and, to a lesser extent, Southeast Asia [27].

In Asia, Africa and the majority of underdeveloped countries in Central and South America, this technology has not contributed much to the fact that it has not been used at all or used appropriately. In 1930s and 1940s non-symbiotic rhizosphere-related *Azotobacteria* were used on large scales in Russia. The application did not reach a conclusion and was later abandoned. In the 1930s, *Bacillus megaterium* was tried to be used for large scale phosphate solubility in Eastern Europe but it was unsuccessful. In the early 1970s, two major breakthroughs took place in plants. The first of these was to find that *Azospirillum* increased the plant growth without directly affecting the plant metabolism, and secondly, the biostimulation agents *Pseudomonas fluorescence* and *Pseudomonas putida* bacteria were being investigated extensively [28].

1.6.2. Genus and Types of Plant Growth Promoting Rhizobacteria

Bacteria that promote plant growth belong to the genus as follows; *Acetobacter, Acinetobacter, Achromobacter, Aereobacter, Agrobacterium, Alcaligenes, Artrobacter,*

Azospirillum, Azotobacter, Bacillus, Burkholderia, Clostridium, Enterobacter, Erwinia, Flavobacterium, Klebsiella, Microccocus, Pseudomonas, Rhizobium, Serratia and Xanthomonas. In recent years, intensive research has been conducted on the use of bacteria such as *Rhizobium, Azotobacter, Bacillus, Azospirillum Pseudomonas, Enterobacter, Klebsiella* and *Staphylococcus* as biological fertilizers and positive results are obtained [29].

The reactions of the symbiotic or non-symbiotic bacterial inoculations by plants depend on the bacterial species, plant species, soil type, bacterial inoculation density and environmental conditions. The major role of the applied bacterial solution is to maintain bacteria density as a result of creating a suitable microenvironment by eliminating the factors that will rapidly reduce bacteria density and then to allow bacteria to enter the soil. Although much is known about how to maintain the viability of bacteria in laboratory conditions, much remains to know about how long the bacteria can withstand natural conditions and stress conditions [30]. In this context, it is important to design bacterial solutions that will be prepared when bacterial inoculation is desired so as to benefit plants by staying alive in soil. The most commonly known PGPR bacteria are shown in Table 1.2.

Table 1.2. Types of plant growth promoting rhizobacteria

Bacteria		
Azorhizobium caulinodans	*Bacillus polymyxa*	*Klebsiella planticola*
Azospirillum amazonense	*Bacillus pumilus*	*Kluyvera ascorbata*
Azospirillum halopraeferens	*Bacillus sphaericus*	*K. cryocrescens*
Azospirillum irakense	*Bacillus subtilis*	*Phyllobacterium rubiacearum*
Azospirillum lipoferum	*Burkholderia cepacia*	*Pseudomonas aeruginosa*

Azospirillum brasilense	*Burkholderia gladioli*	*Pseudomonas aureofaciens*
Azotobacter chroococcum	*Burkholderia graminis*	*Pseudomonas corrugata*
Bacillus cereus	*Burkholderia vietnamensis*	*Pseudomonas fl uorescens*
Bacillus coagulans	*Citrobacter freundii*	*Pseudomonas marginalis*
Bacillus lateroporus	*Curtobacterium flaccumfaciens*	*Pseudomonas putida*
Bacillus licheniformis	*Enterobacter agglomerans*	*Pseudomonas rubrilineans*
Bacillus macerans	*Enterobacter cloacae*	*Rathayibacter rathayi*
Bacillus megaterium	*Erwinia herbicola*	*Serratia marcescens*
Bacillus mycoides	*Flavimonas oryzihabitans*	*Stenotrophomonas sp*
Bacillus pasteurii	*Hydrogenophaga pseudoflava*	*Streptomyces griseoviridis*

1.6.3. Potential Roles of PGPR

PGPRs are mainly divided into two groups; the first is those that are directly effective in plant development, seed output and product yield, while the second group are indirectly useful for plant growth with bio-control [31]. PGPRs promote plant growth through plant development, nitrogen fixation, bioavailability of phosphorus, and removal of iron by plants with siderophore, production of plant hormones such as auxin, cytokinin and gibberellin and reduction of ethylene in plant [32]. In many regions of the world, the use of PGPRs as biological fertilizers has become widespread in order to reduce the potential for industrial fertilizers and pesticide applications [33].

1.6.3.1. Production of Plant Hormone

PGPRs contribute to plant growth indirectly through the production of plant hormones. PGPRs produce herbal hormones such as auxin, cytokinine, zeatine, ethylene, gibberellic acid (GA_3), abscisic acid and ACC Deaminase, which regulate the level of ethylene (Table 1.3).

Table 1.3. PGPRS and plant hormones they produce

Plant hormones	**PGPRs**
Indole-3-Asetic Acid (IAA)	*Acetobacter diazotrophicus, Herbaspirillum seropedicae, Aeromonas veronii, Enterobacter cloacae, Azospirillum brasilense, Enterobacter spp., Agrobacterium spp., Alcaligenes piechaudii, Bradyrhizobium spp., Comamonas acidovorans, Rhizobium leguminosarum*
Cytokinine	*Paenibacillus polymyxa, Pseudomonas fluorescens, Rhizobium leguminosarum*
Zeatine and Ethylene	*Azospirillum spp.*
Gibberellic Acid (GA_3)	*Azospirillum lipoferum, Bacillus spp.*
Abscisic acid (ABA)	*Azospirillum brasilense*
ACC Deaminase	*Bacillus pumilus, Burkholderia cepacian*

1.6.3.2. Biological Nitrogen Fixation

Biological nitrogen fixation is a sustainable source of nitrogen for plant production as a renewable resource. Although a significant amount of fossil energy is used in the production of synthetic nitrogen fertilizers, the energy required by biological nitrogen fixation is provided by photosynthesis and is cost-effective [34]. Therefore, biological

nitrogen fixation is an environmentally friendly approach. PGPRs make it useful for plants by binding free nitrogen in the soil and contribute to plant growth. Nitrogen is one of the basic plant nutrients, it has a heavy loss in the soil with excessive rainfall and mineral washing, and it plays a limiting role in plant growth [35]. Nitrogen is indispensable for every lifestyle and is involved in the synthesis of nucleic acids, proteins and other organic nitrogen compounds [36]. Unfortunately, no plant species has the ability to store nitrogen in the air, so plants use nitrogen in the air directly for growth. Therefore, plants are dependent on nitrogen. PGPRs can keep free nitrogen in the air both symbiotic and non-symbiotic. PGPRs carry the free nitrogen in the air by entering into plant cells symbiotically, form nodules, and provide free nitrogen to the plants. This is often seen in legumes [37]. PGPRs, which supply nitrogen to plants symbiotically are; *Rhizobium, Mesorhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium* and *Sinorhizobium* species. The bacteria cause the fact that the free nitrogen of non-symbiotic air is brought into the plants as they keep the free nitrogen of the air around the root (rhizosphere) which is transformed into an absorbable form by plant roots [38]. The free-living Diazotroph bacteria in the soil take up the role of non-symbiotic free nitrogen acquire into plants. These bacteria are mainly *Azoarcus* [39], *Azospirillum* [40], *Burkholderia* [41], *Gluconacetobacter* [42], *Pseudomonas* [43], *Azotobacter, Arthrobacter, Acinetobacter, Bacillus, Enterobacter, Erwinia, Flavobacterium, Klebsiella, Acetobacter*. Free-living bacteria generally provide 15-200 kg for a hectare per year, *cyanobacteria* 7-80 kg, and *Rhizobium*-legume jointly provide 24-584 kg nitrogen [44, 45].

1.6.3.3. Mineral Phosphate Solubilization

Phosphorus (P) is an essential element that plays an important role in plant growth. Phosphorus intake is inadequate because it is generally insoluble in soil. After application of inorganic phosphate fertilizers to soil, most of them turn into form, which cannot be taken by plants. Applied phosphate fertilizer precipitates as 75-90 percent iron (Fe), aluminium (Al) and calcium (Ca) compounds [46]. The use of dense fertilizer to meet the insufficient phosphorus in the soil causes high cost and environmental problems. Many useful bacteria convert the use of organic acids or other mechanisms into the usable forms for plants by increasing the solubility of inorganic and organic phosphate [47]. Agricultural production has been reported to increase by 10-15 percent due to the use of phosphate

solvent bacteria as biological fertilizers. Plants receive phosphorus in HPO_4 or H_2PO_4 forms. The soil phosphorus must be dissolved in order to be taken by plants and microorganisms. In this context, microorganisms play an important role in the phosphorus cycle. In the soils, mineral phosphate is present in primary, hydroxy and oxy apatite-like minerals. In addition, the soluble P applied as chemical fertilizer is kept in the soil and can cannot be converted into an obtainable form, depending on the pH and type of soil. Studies have shown that bacteria can resolve bound phosphate in P compounds [48].

1.6.3.4. Chelating of Iron by Producing Siderophores

Although iron is one of the abundant minerals on earth, it is not available for plants in soil. Soil Fe is present in oxidized form (Fe^{3+}) as a component of insoluble minerals such as iron hydroxide (FeO (OH)) and iron oxide (Fe_2O_3). To overcome this problem, PGPR secretes siderophores. Siderophores are low molecular weight iron binding proteins and have a high binding ability with iron ions. Some PGPRs produce siderophore that reduces iron for harmful microorganisms and consequently reduce the pathogenicity of these harmful microorganisms [49, 50]. The bacteria living in the rhizosphere, such as *Bacillus*, *Pseudomonas, Geobacter, Alcaligenes, Clostridium* and *Enterobacter*, convert Fe^{2+}, which is a form that plants can take by reducing Fe^{3+} [50, 51]. This chemical reaction occurs as follows:

$$FeO(OH) + 3H^+ + e \leftrightarrow Fe^{+2} + 2H_2O \quad (1.6)$$

$$Fe_2O_3 + 6H^+ + 2e^- \leftrightarrow 2Fe^{+2} + 3H_2O \quad (1.7)$$

1.6.3.5. Producing ACC Deaminase to Regulate Ethylene Level in plants

The importance of ethylene for plants as well as overexpression under stress conditions hampers the development of plants. Ethylene emerges as an indicator of many physiological events in plants such as; the breakdown of dormancy in plants, root formation and elongation, root and body differentiation, leaves and fruit spills, end of flowering, fruit maturation [52]. On the other hand, the production of roots under the conditions of biotic and abiotic stress increases root prolongation and auxin transport, accelerating the aging and shedding of leaves [53]. As a result of the increase in the

amount of ethylene in the roots, root development disorders occur. In order to refine this abnormal condition, the level of ethylene in the roots and production must be restored to normal levels. In this context, 1-aminoclopropane-1-carboxylate (ACC) deaminase plays an important role in normalizing plant growth and development by adjusting the level of ethylene hormone in plants. *Enterobacter sp., Rhizobium sp., Pseudomonas sp., Variovorax sp., Alcaligenes sp.* and *Bacillus sp*. bacterial species reduce the negative effects of ethylene [54]

1.6.3.6. Resistance to Abiotic Stress Conditions

Drought stress limits the yield of agricultural products and plant growth, especially in arid and semi-arid areas. Inactivation of plants by PGPR increases IAA, cytokinin, antioxidants and ACC deaminase production. Increased IAA, cytokinin, antioxidants and ACC deaminase in the plant provide resistance to stress conditions (drought and salinity), while minimizing the effects of plant stressors (Ethylene, ABA, ROS) [55].

1.6.4. Patented Plant Growth Promoting Rhizobacteria

From 1979 to 2017, a total of 273 patents were received and 126 of them were registered. Among these patented PGPRs; 113 were patented by firms, 109 by universities and research stations, 36 by researchers, 3 by firms and universities collaborations, and 12 by firms and researchers. When we look at the countries, China is the first in the number of patents in PGPRs, followed by the USA, Australia, Canada, India, Japan and South Korea (Figure 1.2).

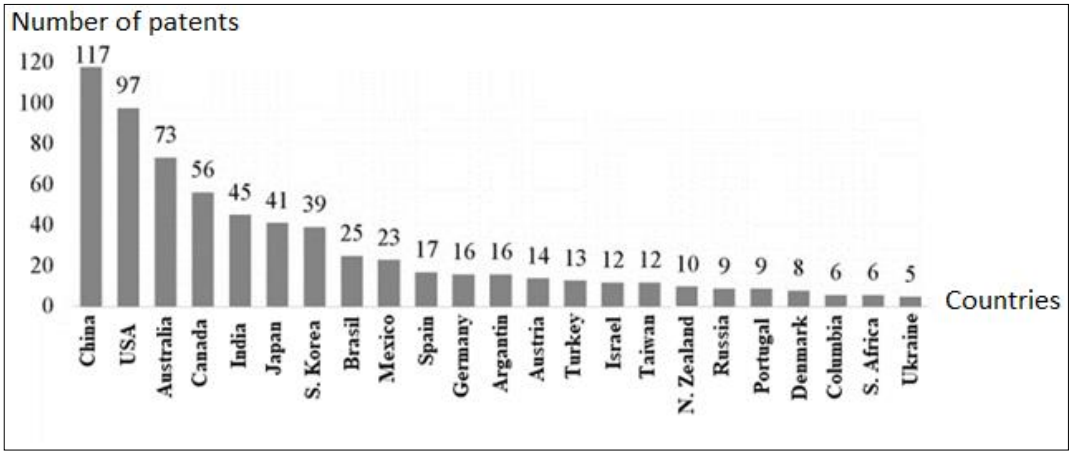


Figure 1.2. The number of PGPR patents among countries [56]

1.6.5. The Effect of PGPRs Against Cold Stress

The microorganisms can be classified as psychrophiles (cold-loving) and psychrotolerant (cold-tolerant) based on their reaction to low (cold) temperatures. Microorganisms have become crucial components of the ecosystem, which arouse interest on their roles in nutrient cycling within cold ecosystems. *Erwinia herbicola* and *Pseudomonas syringae* epiphytic bacteria, which has ice-nucleating activity, in other terms ice positive bacteria, can prevent plants against supercooling by serving as ice nuclei [54]. While such epiphytic PGPRs as well as endophytic types improve their chilling (stress) resistance, they also have enhancing effects on plant growth and productivity. The climate with a wide temperature range as high temperatures in summer and low temperatures in winter, is commonly preferred by psychrotolerant microorganisms to grow and proliferate. This cold-tolerant type of PGPRs are able to promote crop growing even in the coldest parts of the world as they can be metabolically functional to enable nutrient uptake of plants and to produce plant growth regulators [57]. In addition, PGPRs have important roles under cold stress such as inducing pathogen-resistance systems of the host plant, endamaging phytopathogen organization, stimulating phytohormone production and improving management of nutrient and water by plants [57]. Researchers developed a psychrotolerant mutant type of *Pseudomonas fluorescens* which can produce siderophore 17-fold more and increase rhizosphere colonization. The agricultural efficacy of this PGPR was shown on *Vigna radiate* plants between 10 to 25 °C. However, it requires further studies to well-identify

growth promoting effect by siderophore production [58]. Nitrogen fixation role of PGPRs is negatively affected by cold stress, resulting suppression of nodule competitiveness and functioning. In one of the leading studies, the candidate rhizobia (*Mesorhizobium sp, Oxytropis spp.* and *R. leguminosarum*) were examined to overcome cold-stress of plants since they were able to grow at 0°. As a result, *Mesorhizobium sp* had improved legume sainfoin seed growth [59]. Selvakumar and his colleagues had shown that *Exiguobacterium acetylicum* bacterium that was isolated from the Indian Himalayas soil, inhibited the development and growth of infectious pathogens of plants such as *Sclerotium rolfsii, Rhizoctonia solani*, *Fusarium oxysporium* and *Phythium* by *p*roducing siderophores at 4 °C [60]. As described above, P-solubilization is one of the key mechanisms of PGPRs. A mutant type of Pseudomonas was found to show P-solubilization activity at 10°C. As a result, Pseudomonas corrugate facilitated plant growth at low temperature by increasing P-solubilization and organic acid formation [61]. The physiological and growth promoting effects of PGPR on wheat seeds, were studied at 10± 2°C greenhouse conditions. The *Pseudomonas* bacterization significantly improved shoot plant length, root length, decreased Na+/K+ ratio, enhanced anthocyanin, total chlorophyll, total phenolics, free proline, and starch content when compared with seed inoculation in non-bacterized soil condition [62]. These findings were supported by another study in which wheat seed bacterized inoculation (with Pseudomonas sp PPERs23 cold-tolerant type) at low temperature, showed improved root and shooth length, dry root and shoot biomass, total contents of chlorophyll, phenol and aminoacid in leaves at the end of 60 days. Additionally, physiologically available protein and iron concentrations, proline, anthocyanin and relative water content were enhanced while Na+/K+ ratio was decreased in favor of the wheat plants. Eventually, the grain yield was increased 13.4 percent more than condition of unbacterized inoculation [63]. The parameters that were taken into account are quite critical for cold-stress tolerance ability of the plant. According to these studies, the activities maintained by PGPRs against cold-stress are summarized in Figure 1.3.

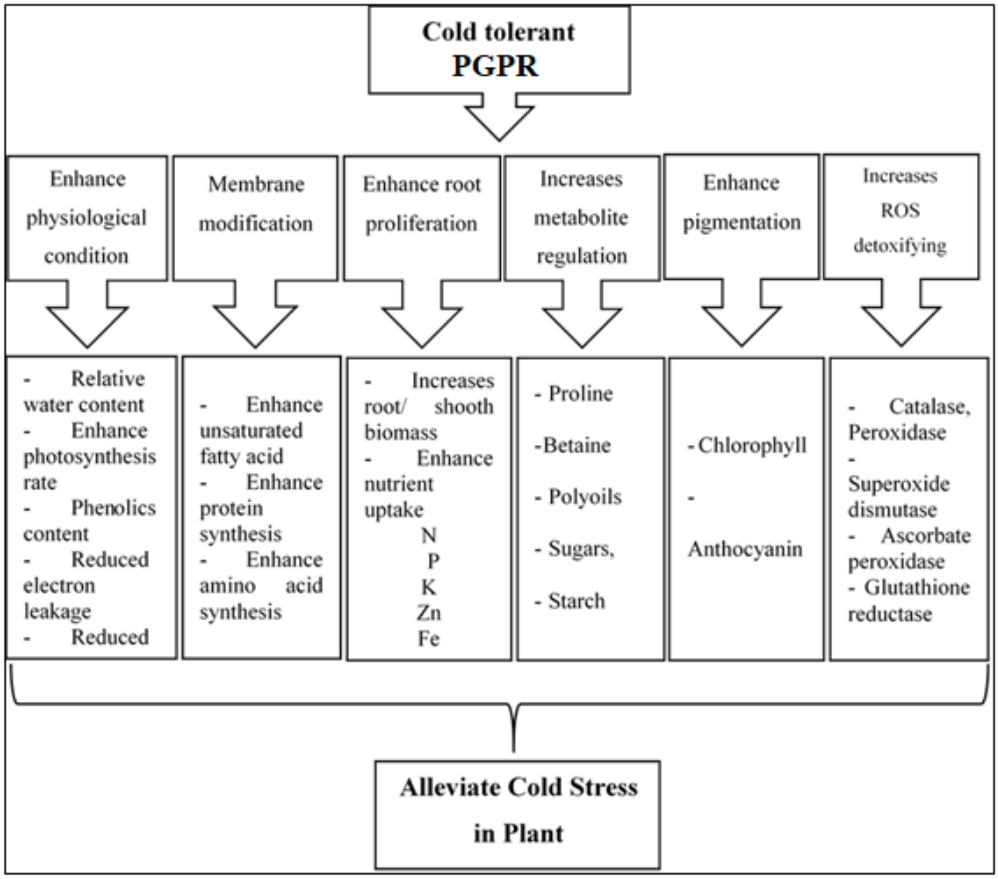


Figure 1.3. The mechanisms of PGPR against cold-stress in plants

1.6.6. *Azosprillium* Bacteria as a PGPR

Azospirillum is a free-living gram-negative bacterium that can fix nitrogen. *Azospirillum* is among the microorganisms that increase plant growth. In 1925, the first species of this genus was isolated and was called *Spirillum lipoferum*. It was not until the year 1978 that the species was proposed as *Azospirillum*. Currently, twelve sub-species of this species are identified: A. *lipoferum and A. brasilense, A. amazonense, A. halopraeferens, A. irakense, A. largimobile, A. doebereinerae, A. oryzae, A. melinis, A. canadense, A. zeae and A. rugosum.* These genera belong to the *Rhodospirillales* order and *Alphaproteobacteria* subclass [39, 64].

It can be easily identified according to the vibrio or thick rod shape, pleomorphism and spiral mobility. They can be straight or slightly curved, with a diameter of about 1 μm and a length of 2.1 to 3.8 1 μm. Generally, the tips are sharp (Figure 1.4).

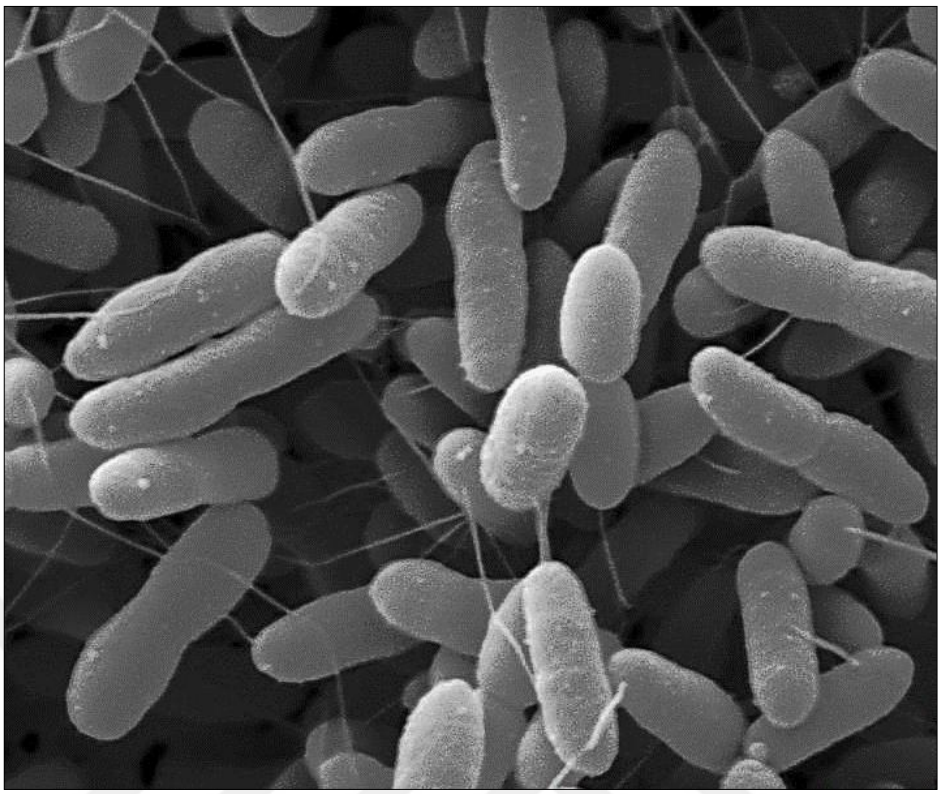


Figure 1.4. *Azospirillum* SEM Photography [65].

Azospirillum polar and lateral flagella patterns show significant mobility. The first flagella group is primarily used for swimming, while the second relates to displacement on solid surfaces. Some species only present polar flagellum. This mobility allows bacteria to move into areas where conditions are suitable for growth. They also offer chemical attraction to organic acids, aromatic compounds, sugars and amino acids. When faced with unfavorable conditions - for example, drying or shortage of nutrients – this bacterium can take cystic forms and develop an outer shell of polysaccharides [64].

Azospirillum is present in the rhizosphere, although there are some species that can affect other areas of the plant, most of them live on the surface of the roots. It has been isolated from different plant species in the world, from tropical climate to temperate regions.

Döbereiner et al. [66] evaluated the effects of Azospirillum on plant growth in two main groups:

i. Increase in plant growth,

ii. Strengthening the root system is to prevent the development of pathogens.

Azospirillum spp. produces phytohormones, such as indole acetic acid, gibberellic acid and cytokinines. They facilitate the plant to obtain mineral substances in the soil [39]. They secrete some signal molecules that pass through the plant cell wall, which are recognized by the plant membrane and cause changes in plant metabolism. In addition, their protective effect against plant pathogens has been shown in the studies [67]. It has been reported that siderophores produced by *A. lipoferum M* isolates exhibit antimicrobial activity against various bacterial and fungal isolates. In a trial conducted in the greenhouse, *A. brasilense Cd* isolate of *Pseudomonas syringae pv*. *tomato* has been reported to protect tomato seedlings against infection [68]. In addition, the application of *Azospirillum* isolates to legumes resulted with increase in the number of nodules, weight and the amount of nitrogen fixed in legumes [39].

1.7. BORON ELEMENT IN THE SOIL

Fertilization is a very important factor in increasing the yield and quality of the products. It is well known that balanced fertilization is very important in plant breeding. However, it is a critical issue that the plants are properly fed to obtain high quality and abundant products. The soil in Turkey which is characterized by high pH, high calcareous, heavy texture and low organic substance content, constitute significant limitations for nutrition of plants with microelements particularly Boron (B). Boron has important roles in plant cell wall durability, membrane integrity, phenol metabolism, transport of carbohydrates, the formation of generative organs, pollen germination and pollen tube growth. Boron element required by the trace amount of trace and toxicity is very close to each other element [69].

The potential of plants to obtain micronutrients varies by the factors such as pH, redox potential, structure, organic substance content, plant species and varieties, soil moisture content, temperature and light. Many researchers have reported that B deficiency that might occur due to the environmental, soil and plant factors, has emerged in a growing range at crop production. It has been shown that B deficiency in plant breeding in different regions of the world, has also become an important nutritional problem in our country [70]. In the study of Sillanpa [71] conducted in Turkey; average B concentration of total 298 samples were detected as 1.10 mg kg-1. According to the trials, B application into the soil resulted with an increased yield of the plant.

In a study conducted by Gezgin et al. [72] determined that Konya soil contain 2.48 ppm B on average. Additionally, they reported that the B content was insufficient (5 ppm) in 26.5 percent of soil samples for beta vulgaris. Güneş et al. [73] reported that when the pH of the soil exceeds 6.5, the interaction with B in the soil has begun. In the study of Torun et al. [74] which was supported by BOREN (National Boron Research Institute), boron deficiency was determined in Adana and Osmaniye regions.

1.7.1. Importance of Boron in Growth and Development of Plants

The importance of B in the growth and development of plants is due to its involvement in physiological and biochemical events [75]. B was found to be involved in the transport of sugars in plant physiology, cell wall synthesis, ligninization, structure of cell wall, carbohydrate metabolism, respiration, indole acetic acid (IAA) metabolism, phenol metabolism and integrity of membranes [76]. In general, there are visible signs of B deficiency in concentrations less than 20-20 mg kg-1 in dicotyledons, 10-20 mg kg-1 in maize (Zea mays) and 10 mg kg-1 in wheat. It has been found that boron contributes to living systems by directly playing a proton donor role and by affecting cell membrane structure and functions. Boron is an element necessary for the growth and development of plants. Cyanobacteria also used B in the nitrogen (N) cycle [77].

Although it is known that B is taken into plants as B(OH)3 by passive absorption, it is also taken as B (OH) 4 via active absorption. Boron plants are transported up to the peaks in xylem transmission pipes. The absorption and transport of B in transmission pipes, are closely related to the water intake of the plant. Therefore, there are significant differences in B intake of plants. Plants show differences due to their soil B purchases. The reason for this difference is that they need different amounts of B to grow. Boron deficiency is expressed in mg B per kilogram of dry weight. The critical value for grapes is 5-10 mg B, 20-70 mg B for dicotyledonous plants, 80-250 mg B for milk-like plants. This difference in plant species results from the differences in cell wall structures. Boron deficiency is common in alkaline soils of temperate regions all over the world and also in alkaline soils of arid regions because strong B washing occurs due to the low adsorption of B(OH)3 in the acid soils and strong B fixation as a result of B(OH)4 adsorption in alkaline soil. B deficiency arises in temperate regions, especially in arid and warm years [78].

Boron deficiency primarily causes damage to growth points; thus, it causes a slowdown in plant growth. Since the movement of the boron within the plant is very little, it cannot move from the older parts to the younger and new developing parts. Therefore, B deficiency first appears in the young parts. Young leaves shrivel, often thicken and acquire dark blue or green colors. The internodes shorten, growth is stunted resulting the plant to have a bushed appearance. As a reflection of the irregularity in the transpiration, the leaves and branches take on a brittle structure that can be easily broken [75]. At the advanced stages of boron deficiency, growth points die, usually growth is negatively affected. The development of bud, flower and fruit decreases or stops completely. In mature leaves, interstitial chlorosis occurs and deformity of foliar is seen. Foliar stems and body thicken. Flower formation regresses, cannot complete the development of buds, pollen become vicious, the seeds have wrinkled and deformed appearance. The number and size of flowers are significantly reduced [78].

In order to increase the efficiency of boron use, it is imperative to determine the application time according to the critical growth periods in which plants need the most. In contrast, the amount of B needed for different plants and their growth periods varies. The amount of B in small grained plants such as wheat and canola are important in generative growth periods rather than vegetative growth periods. However, in root-tuberous plants such as potatoes, tapioca and sugar beet, a long period of time is needed for the transportation of photosynthesis products from the storage to the high-density areas. Therefore, the plants in question need to be provided with continuous B throughout the entire growth period. Recent studies have shown that the adequate presence of B in generative organs is necessary for efficiency and even in fruit trees where there is no indication of B deficiency, the B supplementation increases the yield in various fruit types such as almonds, olives, apples and sour cherries [79]. In California (USA) almond-bred gardens in autumn foliar, B spraying from the leaves has become a common practice. The increase in yield obtained from boron application is mainly due to the high amount of B, which is needed for a temporary period of time during flowering. In this regard, the application of boron from soil and foliage is of great importance in fruit cultivation [78]. In the study aiming to determine the effect of B application, the development of wheat varieties and the distribution of B above the soil were found different. Macaroni varieties were more affected by B than bread varieties. Gezgin et al [80] conducted a study in the

most dense agricultural regions of the Konya Plain (Çumra, Altınekin and Seydişehir), to determine B content, root yield, sugar ratio and sugar yield under three different application ways (soil, foliar, soil+foliar) of various concentrations of B (0, 0.15, 0.30, 0.45 ve 0.60 kg/da). The soil + foliar application has increased the yield significantly.

1.7.2. Boron and Cold Stress of Plants

The determining factor of crop species is the intrinsic chilling sensitivity, in response to boron fractionalization and uptake or transport to root chills. Some tropical and sub-tropical crop species can be grouped as chilling-sensitive, which are *Manihot esculenta* (cassava), *Gossypium hirsutum, Ephedra vulgaris* and *Vigna radiate.* In addition, some are moderately sensitive such as *Cucumis sativum*, *Zeamays*, *Glycine max*, *Sorghum* spp and *Phaseolus vulgaris* [81]. The other group of species which is chilling-resistant or tolerant include *Brassica* spp, *Hordeum vulgare*, *Triticum aestivum* and *Spinacia oleracea* [81].

Under chilling conditions, the water channels in the roots are negatively affected, resulting low external boric acid uptake and supply. The term “negatively” indicates the water absorption and passive B uptake across the root; the boric acid permeating activity of water channels along root cells’ plasma membrane; and finally, the rapid response of water channels against environmental stress [82]. The sensitivity of plant roots against chilling conditions might be enhanced when there is pre-existing B deficiency in young roots. The protein amounts in water channels of the plasma membrane rapidly decrease under conditions of severe B deficiency such as lack of B supply in root tips during an hour [83]. The evidence suggests that there is an association between B-deficiency and foliar damage in crop plants caused by low temperatures. Although it is known that the underlying mechanism of this association is due to restriction of B-uptake capacity of plants, more studies should be conducted to reveal in a multidimensional approach including physiological, biochemical and molecular levels [82]. Figure 1.5. shows root chill-induced changes in plant–water relationships and associated effects on B uptake, transport and partitioning [82].

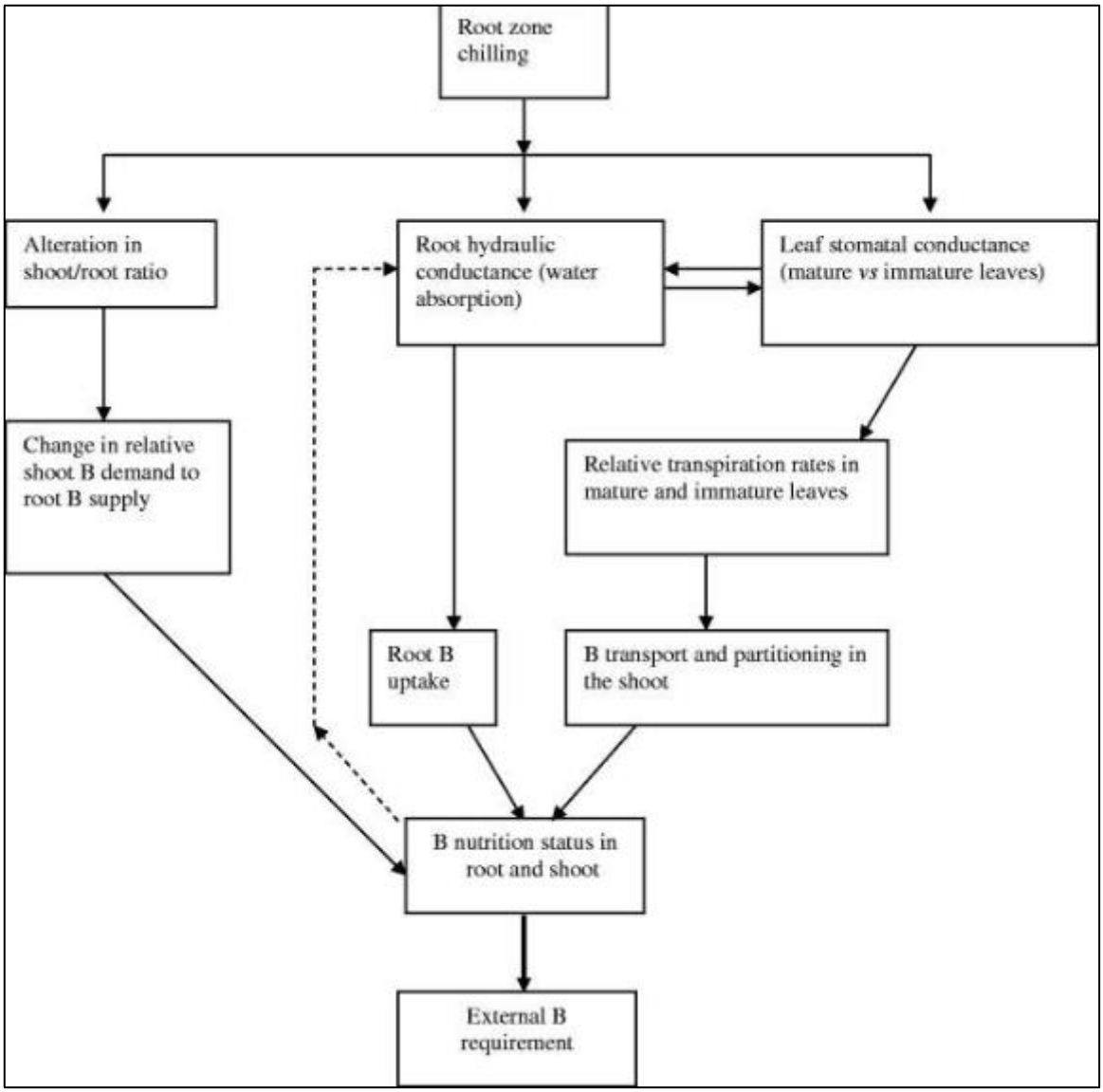


Figure 1.5. The root chill-induced changes in plant–water relationships and associated effects on B uptake, transport and partitioning [82].

1.8. USE OF BORON AND *AZOSPIRILLUM* COMBINATION

The improving effect of *Azospirillum* on nitrogen nutrition of the crops when applied as bio-fertilizer and reducing effect of nitrogen fertilizer need are well-known according to the literature. However, the effect of *Azospirillum* is not clear when combined with secondary nutrients such as boron, zinc or other micronutrients. The combination of *Azospirillum* and magnesium on black pepper plants was found to result with a better yield and maximum gross. This beneficial situation was not only in terms of yield but in terms of

also economical [84]. Based on this limited literature knowledge, the association and beneficial effect of boron and *Azospirillum* including bio-fertilizer should be studied.

The objective of the study was to develop new biological fertilizer formulations by using boron and certain PGPR under laboratory conditions and to apply this new formulation in the field on wheat and corn plant for minimizing negative effect of cold stress and yield losses. For this purpose, hormone, amino acid, organic acid, cell permeability, stoma permeability, chlorophyll content, freezing damage rate in apoplastic area, degree of activity on photosynthesis, apoplastic protein with ice nucleation activity analysis of plant enzymes tests were planned to be performed. In addition wheat plant yield parameters should were tested according to Bio-B use from soil, foliar or in combination.

2. MATERIALS AND METHOD

2.1. BACTERIAL STRAINS

The PGPR strains Bacillus *pumilus, Raoultella terrigena, Azospirillum brasilense, Azospirillum, Erwinia amylovora, Chryseobacterium indologenes, Bacillus subtilis, Burkholderia cepacia ve Azospirillum + Bacilluspumilus* were obtained from Department of Genetic and Bioengineering and Architecture at Yeditepe University Istanbul, Turkey.

2.2. ICE NUCLEATION TEST OF BIO-B FERTILIZER MICROORGANISMS

The bacterial cultures which were diluted in a sterilized test tube with sterile distilled water to a final concentration of 10^8 cfu/ml inside a class-II biosafety cabinet. Test tubes are shown in Figure 2.1.

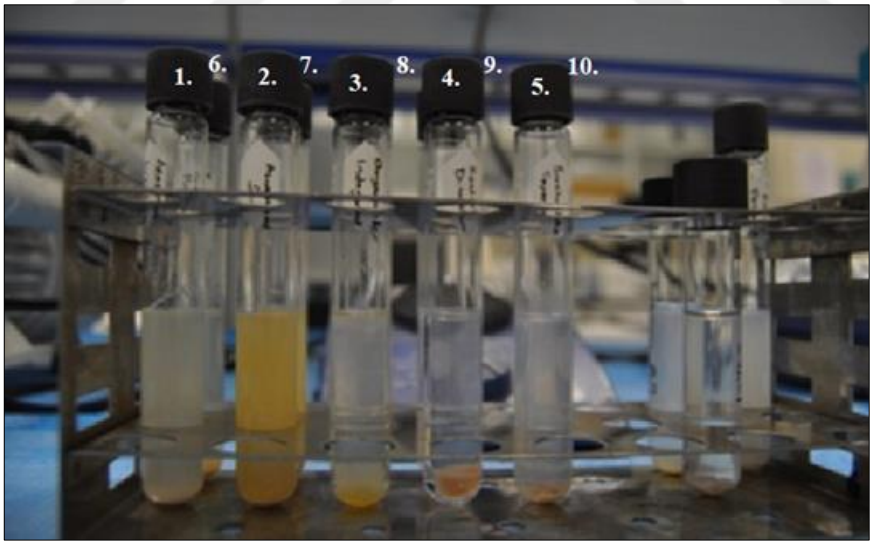


Figure 2.1**.** Ice nucleation activity test tubes. (1.) *Bacillus pumilus,*(2.) *Azospirillum, ,* (3.)*Chryseobacterium indologenes,* (4.) Control, (5.) *Raoultella terrigena,* (6.) *Azospirillum brasilense,* (7.) *Erwinia amylovora,* (8.) *Bacillus subtilis,* (9.) *Burkholderia cepacia* and (10.) *Azospirillum + Bacillus pumilus*

Then, the tubes containing these microorganisms were homogenously distributed in sterile water using vortex. The temperature of the water bath which was prepared before increased to -6°C in this process by adding ice packs with 50 percent ethanol and 50 percent water. Temperature control was adjusted by using thermometer. E.coli which was observed to compose ice nucleus at -6°C was used for a control purpose. Sterile water, which did not contain any microorganisms for control purposes, was used. The experimental setting was prepared by adding equal concentrate microorganisms and equal volume of steril water. Test tubes were checked every 30 minutes.

2.3. *AZOSPIRILLUM BRASILENSE* BIOCHEMICAL REACTION ANALYSIS

Ala-Phe-Pro-arylamidase, adonitol, L-pyrrolidonyl-arylamidase, L-arabitol, D-cellobiose, Beta-galactosidase, H2S formation, Beta-N-acetyl-glucosaminidase, Glutamyl Arylamidase, D-glucose, The gamma-glutamyl-transferase, Fermantation / glucose, Beta-glucosidase, D-Maltose, D-mannitol, D-mannose, Beta-xylosidase, BETA-Alanine arylamidase, L-Proline Arylamidase, Lipase, palatinose, Tyrosine Arylamidase, urease, D-sorbitol, Saccharose / Sucrose, D-Tagatose, D-trehalose, Citrat (Sodium), malonate, 5-Keto-D-Gluconate, L-Lactate, Alpha-glucosidase, Succinate alkalization, N-acetyl-beta-galactosaminidase, ALFA-galactosidase, Phosphate Phos, Glycine arylamidase, Ornitine Dekarboxylazase, Lysine decarboxylase, Decarboxylase Some, L-Histidine assimilation, Beta-glucuronidase, O / 129 resistance (comp.vibrio.), Glu-Glu-Arg-arylamidase, L-Malat Assimilation, Ellman Ellm, L-Lactate assimilation biochemical reaction tests were conducted for *Azospirillum brasilense.* Test results were given as “negative” or “positive”.

2.4. FLUORESCENCE STAINING OF *AZOSPIRILLUM BRASILENSE*

Another method is the fluorescence staining method. In this method, some microorganisms are either self-irradiation at some wavelengths or radiate in some color spectrum. Thus, microorganism dimensions and morphological images are obtained. For this purpose, after staining with FITC (green fluorescent), images were taken by phase contrast invert fluorescent (Zeiss brand) microscope.

2.5. *AZOSPIRILLUM BRASILENSE* GRAM STAINING TEST

Gram staining is one of the most useful staining techniques in the bacteriology laboratory. This technique was used to distinguish Gram-positive / Gram-negative as the first step in the identification of a bacterial specimen.

i. Gram staining procedure:

- The crystal violet dye was left on the slide, prepared for Gram staining for 1 minute and washed away with iodine-lugol solution.
- Iodine-lugol solution was re-added dropwise to the slide for 1 - 2 minutes and washed with distilled water to remove the iodine-lugol solution.
- A solution of 96 percent ethyl alcohol or ether - acetone solution was added dropwise over 15 - 30 seconds.
- Washed with distilled water and safranin was dropped as the opposite dye and leave for 40 - 50 seconds.
- The mixture was washed with distilled water and allowed to dry in the air.
- Immersion oil was added to the preparation and examined with a 100x lens. Purple colored bacteria were evaluated as Gram positive and pink-red colored bacteria as Gram negative.

ii. Staining procedure with KOH solution:

This method was also used in order to understand whether the bacterial strain we have was gram positive or gram negative. The following procedure was used as a practical procedure.

- 3 percent KOH solution was placed dropwise in the petri dish.
- A quantity was taken from the bacterium and mixed with the solution.
- The needle was raised from the drip. If KOH becomes viscose and prolongs like a 0.5-2 cm or higher thread, this meant a positive reaction and occurred in gram-negative bacteria. If an aqueous liquid was formed and did not grow with the core, this result was a negative reaction and occurred in gram-positive bacteria.

2.6. GENOMIC DNA ISOLATION FROM MICROORGANISM FOR POLIMERASE CHAIN REACTION AMPLIFICATION

The DNA isolation of the bacterial strain, was done by the help of mini kits prepared by the companies as follows.

- 2 water baths were set to 37 ºC and 55ºC.
- 1 µl of Lysozyme Digestion Buffer was taken into the falcon tube.
- Then 30 mg fresh Lysozyme was added and mixed until dissolved.
- Tubes were labeled. 180 µl from the mixture was placed in tubes. With the help of a microbiological needle, some number of bacteria were placed in tubes.
- Tubes were kept in a 37 ºC water bath for half an hour.
- 20 µl of Proteinase and 200 µl of Genomiclysis / Binding buffer were added respectively.
- It was left in the 55 ºC water bath for 30 minutes.
- Then 200 µl of 96-100 percent ethanol was added. The obtained lysates were transferred to special tubes which are called spincolumn.
- Centrifuge at the latest speed for 1.5 minutes.
- 500 µl Washbuffer1 supplied with the kit was added to the columns.
- Centrifuged at room temperature for 1 min. The bottom of the tube was discarded.
- Again 500 µl of Wash buffer2 was added. Centrifuged for 3 minutes. The bottom of the tube was re-centrifuged again for 1 minute after it was removed again.
- The tubes remained under the lids were replaced. 500 µl of Genomic Elution Buffer was added to each tube. Incubated for 1 minute at room temperature. The bacterium DNA, which was now pure in the tube, was obtained.

The concentration of the resulting amount of DNA was measured by the Nanodrop device. First, we placed 1 µl of buffers on the machine for the “blank”. We pressed the Blank button. Then we placed the sample we had. The machine automatically gave us the

concentration of the sample in the form of ng / ml. We could achieve the desired concentration with the following formula.

$$D = \frac{W \times V}{A} \quad (2.1)$$

D: DNA working concentration (ng/ml)

W: wanted concentration (50 ng/ml)

V: volume intented to be prepared (200 ml)

A: existing DNA concentration of the sample (ng/ml)

2.7. POLIMERASE CHAIN REACTION (PCR)

We put ice into a container. Matermix required for PCR was placed in ice. The amounts and solutions required are shown in Table 2.1.

Table 2.1. Required content and amounts for PCR

Content	**Amount**
2 × Mastermix	10µl
Template DNA	1-2 µl
Primer f	1 µl
Primer r	1 µl
d H_2O	6-7 µl

Primer Name: 1492 R-27 S

Initial incubation: 2 min at 94 ºC,

Each PCR cycle (Denaturation, Primer binding and Amplicon synthesis)

For denaturation 20 sec at 94 ºC,

Primary bonding (Annealing), 20 sec at 50-65 ºC,

Amplicon synthesis (extention), 1 min at 67-72 ºC,

PCR completion incubation at 2-5 min at 72 ºC.

2.8. ELECTROPHORESIS OF PCR AMPLIFICATIONS

- 40 ml of 1 × Tris-Acetate-EDTA (TAE) solution was taken and 0.4 g agarose was added. The microwave was heated to boiling point for 4 minutes. Then etidium bromide was added into the cooled agarose gel to 60ºC. After that, gel was spread to the electrophoresis gel cuvette.
- The frozen gel was placed in the horizontal electrophoresis tank.
- The DNA marker was loaded from the DNA loading wells on the agarose gel to the first and last. One of the other wells, there was negative control and the others loaded with PCR amplicons.
- The electrophoresis gel assembly was set at 130 volts and the samples ran for 3 h on gel.
- The resulting bands were visualized by the gel documentation method under UV.

2.9. MICROBIAL IDENTIFICATION SYSTEM (MIS ANALYSIS)

Microbial Identification System is a rapid phenotypic method for the identification of plant pathogenic bacteria based on gas chromatography analysis of cellular fatty acids (GC-FAME).

This system is based on the number, variety, and percentage of fatty acids in the cells of genetically identical microorganisms (fatty acid profile) being the same and not changing as long as the environmental conditions are the same.

Required extract preparation for MIS:

- Collection: Cells were collected from the culture medium. This was done by collecting the microorganism from the petri dish with the sterile needle.
- Saponification: The process of breaking down the cells to release fatty acids from cellular oils. For this;

i. Reagent 1 (sodium hydroxide + methanol + di-ionized distilled water) was added to a tube.

ii. Mixed for 5-10 seconds with tube mixer.

iii. Waited for 5 minutes at 100 ºC. Stirred again for 5-10 seconds.

iv. Waited for 25 minutes at 100 ºC.

- Methylation: Formation of methyl esters of fatty acids. For this;

i. 2 ml of Reagent 2 (hydrochloric acid + methanol) was added to the tube.

ii. Mixed for 5-10 sec.

iii. Waited at 80 ºC for 10 minutes.

- Extraction: The process of transferring fatty acid methyl esters (FAME) from the aqueous part to the organic part. For this;

i. 1.25 ml of Reagent 3 (hexane + methyltetbutyl ether) was added.

ii. Centrifuged for 10 minutes.

iii. The lower phase was removed and the upper phase was collected.

- Washing: It was the process of washing organic extract before chromotographic analysis. For this process;

i. 3 ml of Reagent 4 (sodium hydroxide + di-ionized distilled water) was added.

ii. Centrifuged for 5 minutes.

iii. 2/3 of the upper phase was collected and transferred to the gas chromatography vial and closed.

2.10. DETERMINING THE EFFECT OF BORON ON *AZOSPIRILLUM BRASILENSE*

The effects of boron amount applied on Bio-B fertilizer formulations with different boron content (control [0.0 percent], 0.1 percent, 0.5 percent, 1.0 percent, 5.0 percent and 10.0 percent boron) have been investigated to determine the effects on *Azospirillum brasilense* viability. The bacteria were inoculated into different concentrated boron containing petri dishes. The colony formings (colony forming unit per ml, CFU/ml) were observed, recorded and evaluated in terms of viability.

2.11. DETERMINING FREEZING POINT

The analyzes were carried out to determine the freezing point of *Azosprillium brasilense* prepared at different boron concentrations. Freezing was also monitored by evaluating morphological changings (shrinking of microorganisms).

The boron concentrations were: control, 0.1 percent, 0.5 percent, 1.0 percent, 5.0 percent and 10.0 percent boron

The temperatures were: 0 ºC, -5 ºC, -10 ºC -15 ºC, -20 ºC, -25 ºC

2.12. INVESTIGATION OF THE ANTAGONISTIC EFFECT OF B FERTILIZER FORMULATIONS ON *PSEUDOMONAS SYRINGAE*

The effects of boron content applied with Bio-B fertilizer formulations including various boron amounts on the viability of *Pseudomonas Syringae* were investigated. For this purpose;

• 0.5-liter Tryptic strain broths (TSB) were prepared as medium in *Pseudomonas Syringae* medium. The prepared media were autoclaved and sterilized at 121 ° C for 15 min.

• Bacteria were placed in the medium and incubated for 1.5 days at 30 ºC.

• Boric acid was then prepared in order to apply boron in different ratios of 0.1 to 10 percent. The amounts of boron and percentages were as shown in Table 2.2.

Table 2.2. Amounts and percentages of boron

Boron percent	Boron amount (g)
0	0
0,2	0,007
0,5	0,01425
1	0,02857
5	0,1428
10	0,2857

• Following the addition, *Pseudomonas Syringae* microorganisms (as in 5 percent inoculant form) were transferred to the medium, mixed and shakened to mix well.

• The prepared mixture was then allowed to incubate again for 1.5 days at 30 ºC.

• At the end of 1.5 days, the absorbance value was read with the sprectrophotometer.

• Afterwards, inoculation was carried out by means of the dilution method and then by means of dragalski to Tryptic soy agar (TSA).

• After incubation for 1 day at 30 ºC, viability was checked.

2.13. FIELD RESEARCH

2.13.1. Wheat

In Erzurum, the seed cultivation of cottage (kırık) wheat varieties was made according to the test pattern depending on the chance with the help of 17-row fertilizer combination with including 10 cm and 34 cm (17x2) spaces in the order of 180 kg seed per hectare, parcel length 7m and parcel width 4 cm (10 cm x34 = 340 cm) was planted in a plot of 28 m^2. Two meters distance between each parcel was left. Four groups which were control, soil, leaves and soil + leaves were applied to these parcels and bio-B fertilizers were

carried out in a total of 12 parcels. 8 to 10 kg N, 12 to 14 kg P_2O_5 and 8 to 10 kg K_2O were applied to the 1-zone area in order to maintain the normal development of the wheat plant by creating trial areas (Figure 2.2-2.3).

Figure 2.2. The area of cultivated wheat according to various groups

Figure 2.3. The area of cultivated wheat according to various groups

The water requirement of the plant was taken in consider while 3 applications were performed in the first weeks of June, July and August. At the end of September, the plants were harvested and yield parameters were determined and samples were brought to the laboratory for the hormone, amino acid, organic acid, cell permeability, stoma permeability, chlorophyll content, freezing damage rate in apoplastic area, degree of activity on photosynthesis, apoplastic protein with ice nucleation activity tests and analysis of plant enzymes and bio-B formulations. In addition, macro and micro elements were determined in soil samples.

2.13.1.1. The Chemical Characteristics of the Soil that Wheat was Cultivated

Soil reaction (pH): The soil-water suspension with 1:2.5 ratio was measured potentiometrically by pH meter with glass electrode (Table 2.3.) [85].

Lime in a soil (CaCO3): It was determined with Scheibler Calcimeter volumetrically [86].

Soil Organic Matter: Soil organic matter was determined by Smith-Weldon method [87].

Determination of soluble Phosphorus: After soils are extracted with sodium bicarbonate ($NaHCO_3$, pH balanced to 8.5), It was determined by reading ICP OEST [88].

Exchangeable Cations: Exchangeable cations of soils were determined by shaking and extracting with Ammonium Acetate and reading by ICP OES spectrophotometer [89].

Micro Element (Fe, Mn, Zn, Cu) Determination: It is determined by reading on ICP OES spectrophotometer in the extracted filtrates according to DTPA method [90].

B Used for Plant: It was determined by reading on ICP OES (Optima 2100 DV Perkin Emler) spectrophotometer in filters extracted with 0.01 M Mannitol +0.01 M $CaCl_2$ solution [91].

2.13.1.2. Effect of Bio-B Fertilizer on Wheat Plant's Yield and Yield Parameters

Wheat plant samples were taken with the help of 50x50 cm square frame from each of the experiment plots which were established with three replications. For this purpose, the ground parts of the wheat plant in the square frame thrown into the trial plot were harvested and the measurement was made for the yield evaluation. The measured values were converted to decares and the changes in the yield of wheat plant due to Bio-B application were determined. In addition, 10 plants were randomly extracted from soil. The yield and yield parameters of wheat plants were examined by bringing the wheat plant's harvested above ground parts to the laboratory. The yields of the plants harvested from the parcels where each treatment was applied were determined. In addition, plant samples from each treatment were dried in air and then allowed to dry in a 68ºC oven for 24 hours. Plant samples' macro and micro element analyzes were carried out by crushing which dry weights were determined in porcelain mortar. For amino acid, organic acid, hormone and enzyme and other biochemical analyzes, samples were taken while the plants were green during the spike and samples were placed in freezer set to -80 ^{0}C until analysis.

Chlorophyll Content: Chlorophyll content of plant leaves were determined by chlorophyll meter with SPA-502 (SPAD-502, Konica Minolta Sensing, Inc., Japan).

Stoma Permeability: The stoma content of the plant samples was determined by measuring with SC-1 poremeter.

Membrane permeability: Leaves membrane permeability test were done according to Lutts et al [92].

Photosynthesis Measurement: In order to determine the physiological processes in plants caused by frost stress, photosynthesis measurements were performed with Li-COR photosynthesis meter following frost stress.

Freeze Damage Rate in Leaves: It is determined according to Griffth et al [93]. Fresh wheat leaves were cut into 2cm lengths and flushed in sterile water. Leaves (0.5g) were placed in test tubes, and then were stuated in a freezing bath. After stabilization at –1°C for 30min, the temperature was brought down that stepwise by 1°C times from –1 to –20°C. The tubes were evacuated, and 4 mL of cold sterile water was included within the test tube. These test tubes were stored at 4°C for 24h.

Determination of the Effect of Ice Nucleation Activity of Apoplastic Proteins: Determination of ice nucleation activity of apoplastic proteins were done according to Griffth et al. [93].

Antioxidant Enzyme Activity in Plants: It was determined by reading the supernatants obtained as a result of extraction processes in spectrophotometer [94].

Amino acid content in plants: Determination of amino acid was determined by reading in HPLC by column separation with phenyl isothiocyanate (PITC) [95].

Amount of Organic Acid in Plants*:* The amount of organic acid in plants was obtained by reading the extraction of fresh tissues in HPLC.

Hormone Determination of Plants*:* Gibberellic acid, indole acetic acid, salicylic acid and abscisic acid analyzes of wheat plants were determined by reading in HPLC [96- 98].

Amount of N in Plant: Nitrogen content of plant samples was determined by micro kjheldahl method after being subjected to wet burning with salicylic-sulfuric acid mixture [99].

Other elements in plants (P, K, Ca, Mg, Na, Fe, Mn, Zn, Cu, B): P, K, Ca, Mg, Na, Fe, Mn, Zn, Cu, and B contents of plant samples were determined by reading ICP OES [100].

3. RESULTS

3.1. ICE NUCLEATION TEST RESULTS OF BIO-B FERTILIZER MICROORGANISMS

In the first two hours only *Erwinia amylovora* 234-1 coded bacteria were observed. The results obtained at the end of the second day are given in Table 3.1. The freezing images of microorganisms are shown in Figures 3.1, 3.2, 3.3, 3.4 and 3.5.

Table 3.1. Ice nucleation test results of microorganisms used in the experiment

Microorganisms **Control duration**	**Frozen tubes**	**Frozen tubes**	**Frozen tubes**	**Frozen tubes**	**Frozen tubes**	**Frozen tubes**
	30th min	1st hour	2nd hour	4th hour	1st day	2nd day
Bacillus pumilus	-	-	+	+	+	+
Raoultellaterrigena	-	-	+	+	+	+
Azospirillum brasilense	-	-	-	-	-	-
Azospirillum	-	-	-	+	+	+
Erwinia amylovora 131	-	-	-	+	+	+
Chryseobacterium indologenes	-	-	+	+	+	+
Bacillus subtilis	-	-	-	+	+	+
Burkholderia cepacia	-	-	-	-	+	+
Azospirillum+ Bacillus pumilus	-	-	-	-	+	+
(Control)D.water	-	-	-	+	+	+

It was observed that the microorganisms did not freeze at -10 degrees during the periods indicated with (-) in the table.

Figure 3.1. Images of (1.) *Bacillus pumilus* and (2.) *Azosprillium + Bacillus pumilus*

Normally, at the end of the second hour, when *the Bacillus pumilus* was frozen at –10 degrees, however there was no changings at the combined sample.

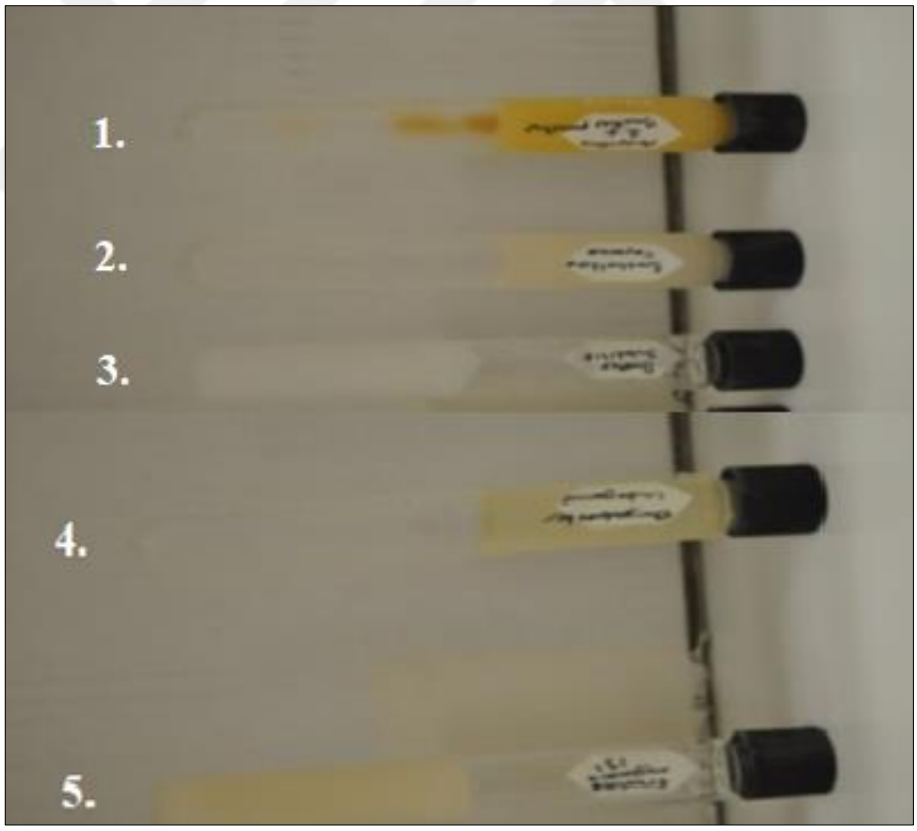


Figure 3.2. Images of four hours after the end of (1.) *Burkholderia cepacia*, (2.) *Erwinia amylovora* 131, (3.) *Bacillus subtilis (4.) Chryseobacterium indologenes* and (5.) *Bacillus pumilus + Azospirillum* after four hours

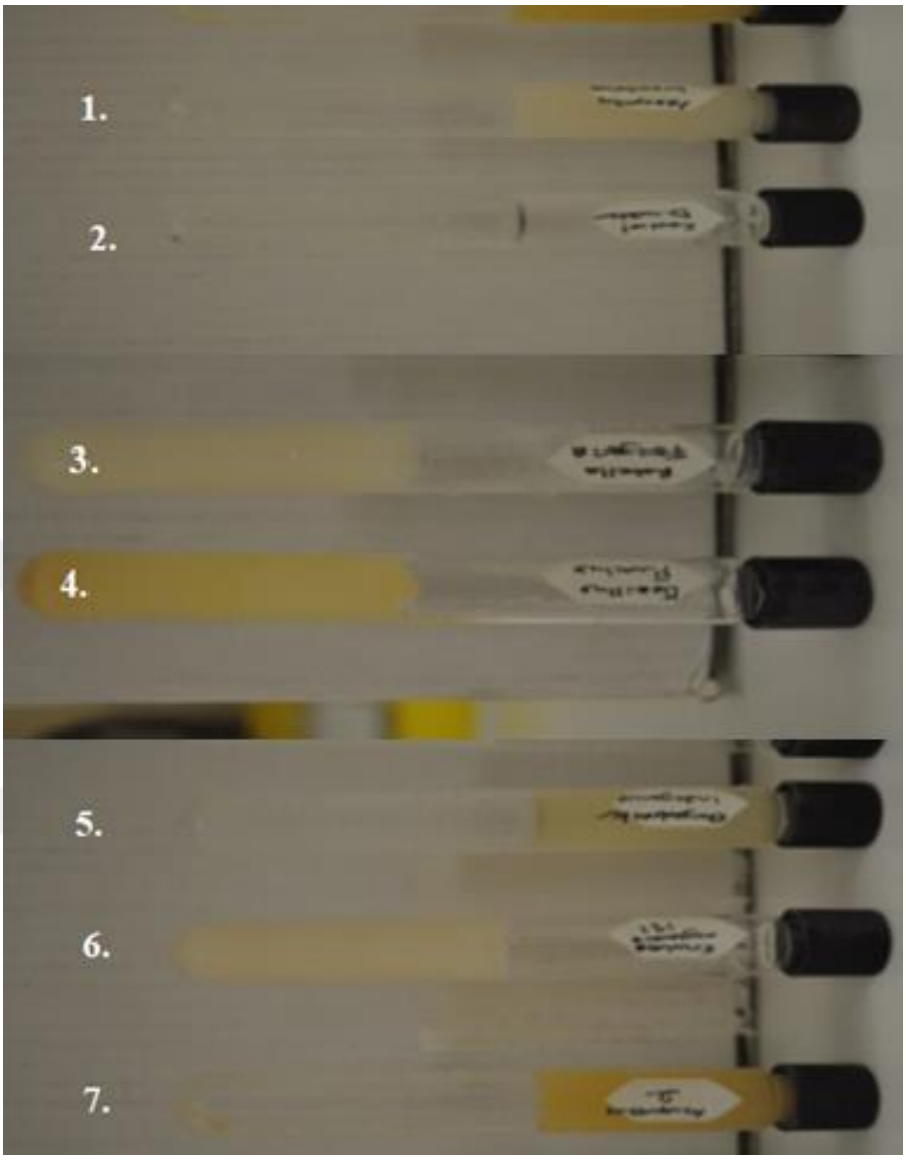


Figure 3.3. Images of (1.) *Azospirillum brasilense*, (2.) Control, (3.) *Raoultella terrigena*, (4.) *Bacillus pumilus*, (5.) *Chryseobacterium indologenes*, (6.) *Erwinia amylovora* (7.) *Azospirillum* 131, after four hours

Figure 3.4. Images of (1.) *Azosprillium + Bacillus Pumilus* after one day

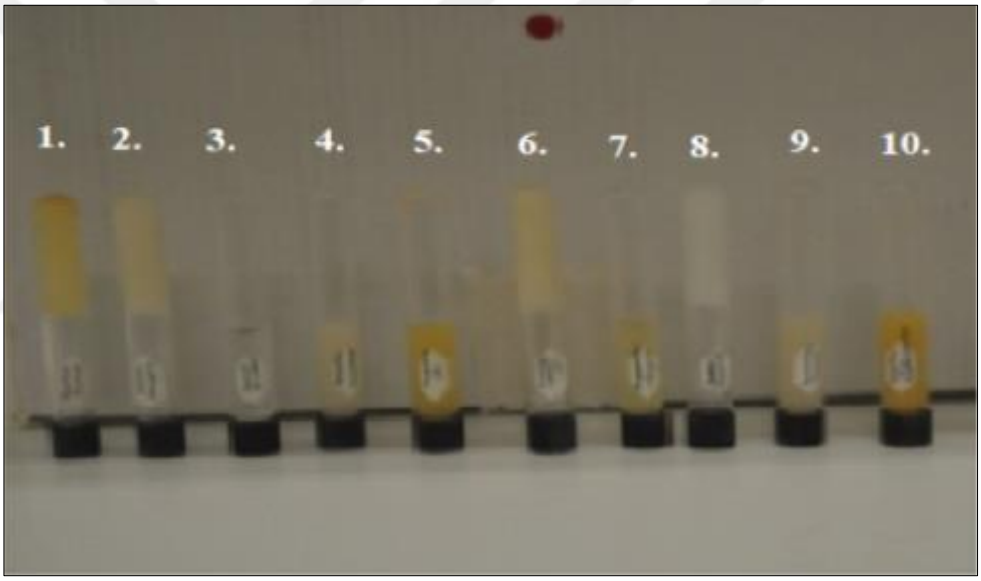


Figure 3.5. Images of (1.) *Bacillus pumilus,* (2.) *Azospirillum brasilense,* (3.) Control, (4.) *Raoultella terrigena,* (5.) *Erwinia amylovora,* (6.) *Azospirillum,*(7.) *Chryseo bacterium indologenes,* (8.)*Burkholderia cepacia, (9.)Bacillus subtilis, (10.)Azospirillum + Bacillus pumilus* and after one day.

At the end of the second day, *Azosprillium Brasilense* was removed from -10 degrees and no freezing was observed.

3.2. *AZOSPIRILLUM BRASILENSE* BIOCHEMICAL REACTION ANALYSIS RESULTS

Microorganisms are defined according to their biochemical behaviors. In order to confirm that the plant promoting ability of *Azospirillum brasilense*, biochemical analyzes were done. The biochemical test results of *Azospirillum brasilense* is as shown in Table 3.2.

Table 3.2. *Azospirillum brasilense* biochemical reaction analysis results

Name of the test	Result	Name of the test	Result
Ala-Phe-Pro-arylamidase	Negative	CITRAT (SODIUM)	Negative
Adonitol	Positive	Malonate	Negative
L-pyrrolidonyl-arylamidase	Positive	5-Keto-D-Gluconate	Negative
L-arabitol	Negative	L-LACTATE	Negative
D-cellobiose	Positive	Alpha-glucosidase	Negative
Beta-galactosidase	Positive	SUCKSHIP alkalization	Negative
H_2S FORMATION	Positive	N-acetyl-beta-galactosaminidase	Negative
Beta-N-acetyl-glucosaminidase	Positive	ALFA-galactosidase	Negative
Glutamyl Arylamidase	Negative	PHOSPHATE PHOS	Negative
D-glucose	Positive	Glycine ARYLAMIDASE	Negative
The gamma-glutamyl-transferase	Negative	ORNITINE DECARBOXYLASE	Negative
		LYSINE DECARBOXYLASE	Negative
FERMANTATION/GLUCOSE	Positive	DECARBOXYLASE SOME	Negative
Beta-glucosidase	Positive	L-HISTIDINE assimilation	Negative
D-Maltose	Positive	KURMARAT	Positive
D-mannitol	Positive	Beta-glucuronidase	Negative
D-mannose	Positive	O/129RESISTANCE	Negative

Beta-xylosidase	Negative	(comp.vibrio.)	Negative
BETA-Alanine arylamidase	Negative	Glu-Glu-Arg-arylamidase	Negative
L-Proline ARYLAMIDASE	Negative	L-MALAT assimilation	Negative
LIPASE	Negative	ELLMAN ELLM	Negative
Palatinose	Negative	L-LACTATE assimilation	
Tyrosine ARYLAMIDASE	Positive		
urease	Negative		
D-sorbitol	Positive		
SACCHAROSE / SUCROSE	Positive		
D-Tagatose	Positive		
D-Trehalose	Positive		

3.3. FLUORESCENCE STAINING RESULTS OF *AZOSPIRILLUM BRASILENSE*

Fluorescence staining of the *Azospirillum brasilense* result is shown in Figure 3.6. It is obtained from the image that how microorganism takes place in a plant's parts and also it gives information about microorganism dimensions and morphological structure.

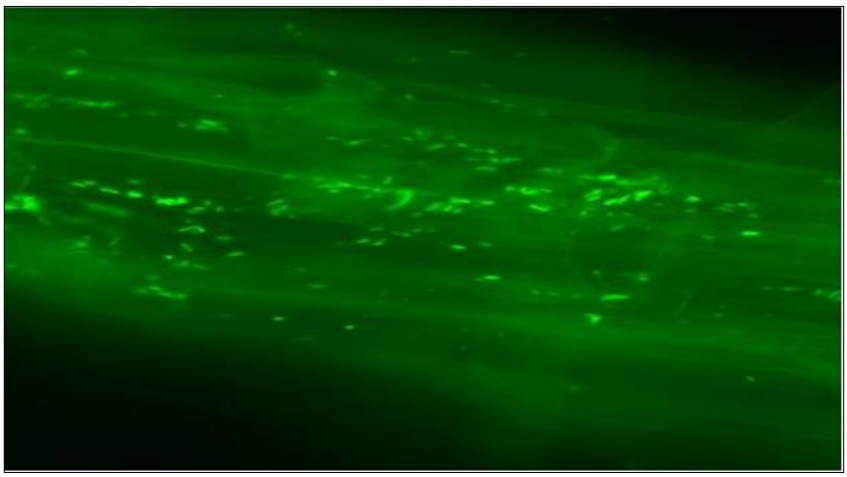


Figure 3.6. FCM image of *Azospirillum brasilense* (63x15; FITC)

3.4. PCR GEL IMAGE OF *AZOSPIRILLUM BRASILENSE*

After gel documentation, the resulting bands were visualized as in Figure 3.7. 16S r RNA gene part is obtained from *Azosprillium brasilense* successfully. The bands were approximately 1500 base pair long.

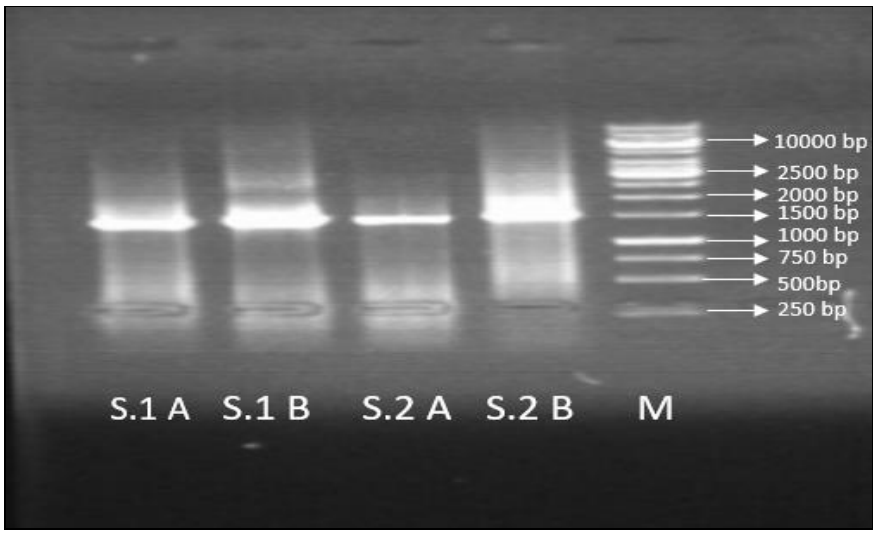


Figure 3.7. PCR gel image of *Azospirillum brasilense* S.1 A: Sample 1 for 50 ^{0}C annealing temperature, S.1 B: Sample 1 for 65 ^{0}C annealing temperature, S.2 A: Sample 2 for 50 ^{0}C annealing temperature, S.2 B: Sample 2 for 65 ^{0}C annealing temperature, M: Marker.

3.5. MIS ANALYSIS RESULTS

After obtaining extracts for target microorganism, the data and graphs are taken from GS-MS machine (Figure3.8). It is understood from the figure that obtained extract are belong to *Azospirillum brasilense* bacterium.

Volume: DATA File: E145125.95A Samp Ctr: 7 ID Number: 9658
Type: Samp Bottle: 6 Method: RTSBA6
Created: 5/12/2014 3:07:31 PM
Sample ID: 2

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
0.6941	880938	0.006	----	6.6644		----	< min rt	
0.7043	9.415E+8	0.020	----	6.7407	SOLVENT PEAK	----	< min rt	
1.7658	338	0.009	1.022	12.9559	13:1 at 12-13	1.01	ECL deviates -0.002	
2.5155	1626	0.008	0.935	15.5122	Sum In Feature 2	4.44	ECL deviates -0.003	14:0 3OH/16:1 iso I
2.6146	4516	0.009	0.928	15.8311	Sum In Feature 3	12.23	ECL deviates -0.009	16:1 w7c/16:1 w6c
2.6653	2760	0.009	0.925	15.9940	16:0	7.45	ECL deviates -0.006	Reference -0.012
3.1503	1248	0.009	0.902	17.5431	16:0 3OH	3.29	ECL deviates -0.005	
3.2420	26009	0.009	0.899	17.8359	Sum In Feature 8	68.25	ECL deviates -0.012	18:1 w7c
3.6401	1291	0.010	0.886	19.1348	18:1 2OH	3.34	ECL deviates -0.011	
----	1626	---	----	----	Summed Feature 2	4.44	12:0 aldehyde ?	unknown 10.9525
----	-----	---	----	----		----	16:1 iso I/14:0 3OH	14:0 3OH/16:1 iso I
----	4516	---	----	----	Summed Feature 3	12.23	16:1 w7c/16:1 w6c	16:1 w6c/16:1 w7c
----	26009	---	----	----	Summed Feature 8	68.25	18:1 w7c	18:1 w6c

ECL Deviation: 0.008 Reference ECL Shift: 0.012 Number Reference Peaks: 1
Total Response: 37787 Total Named: 37787
Percent Named: 100.00% Total Amount: 34263
Profile Comment: Total response less than 50000.0. Concentrate and re-run.

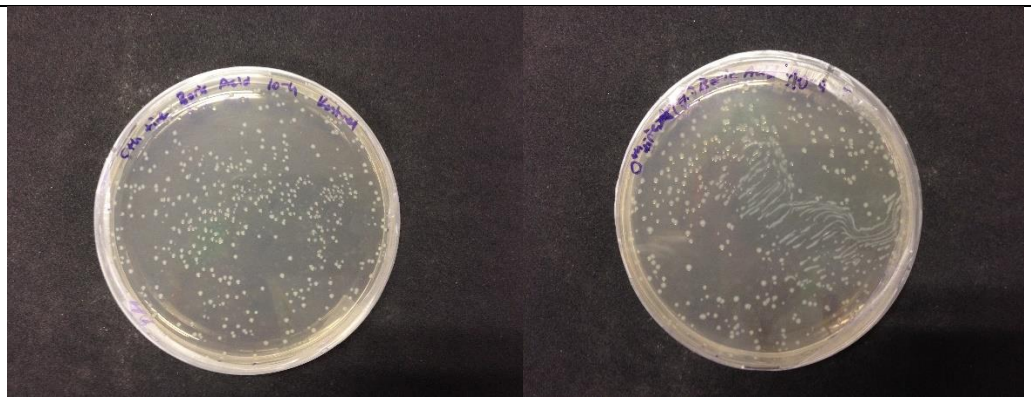
Matches:

Library	Sim Index	Entry Name
RTSBA6 6.00	0.947	Roseomonas-fauriae
	0.614	Azospirillum-brasilense

Figure 3.8. MIS Analysis data and graph of *Azospirillum brasilense*

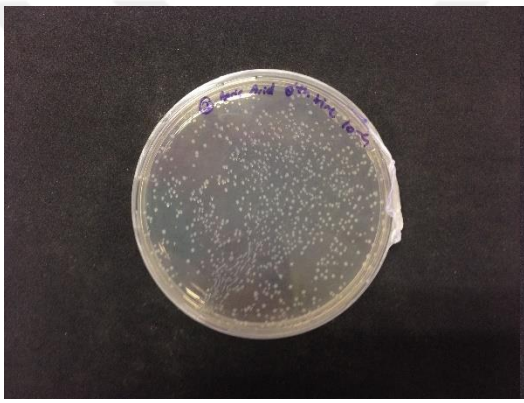
3.6. EFFECT OF DIFFERENT CONCENTRATIONS OF BORIC ACID ON *AZOSPIRILLUM BRASILENSE*

The colonization of *Azospirillum brasilense* bacteria was seen the most at 0.5 percent boron administration. The observed colonization was alike with each other, which was confirmed by CFU/ml quantitative results shown in Table 3.3 and graph in Figure 3.9 and Figure 3.10.

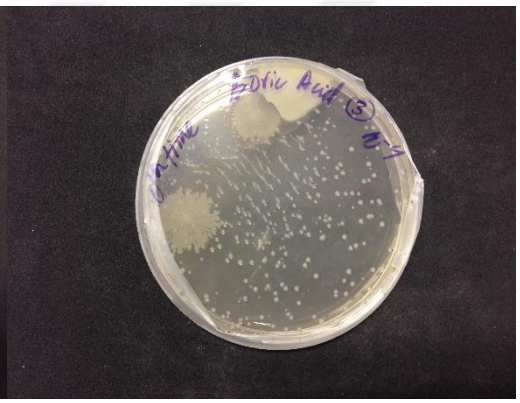


Control

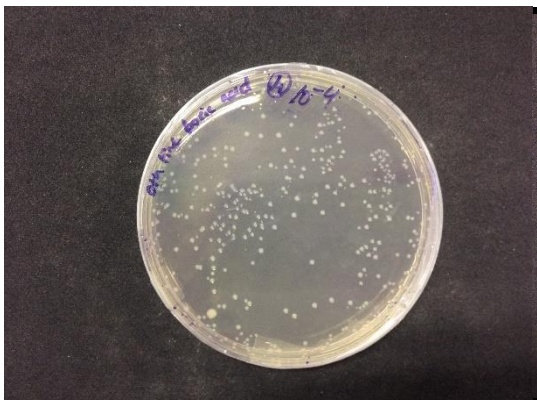
0.1 percent boron



0.5 percent boron



1 percent boron



5 percent boron

10 percent boron

Figure 3.9. The effect of various boron concentration on the colonization and viability *Azospirillum brasilense* bacteria

Table 3.3. The effect of boron on the development of *Azospirillum brasilense* bacteria

Boron percent	Gram	Number of bacteria
0 (control)	0	5.02E+6
0,1	0,011	8.19E+6
0,5	0,029	1.143E+7
1	0,057	3.34E+6
5	0,286	4.32E+6
10	0,571	3.20E+6

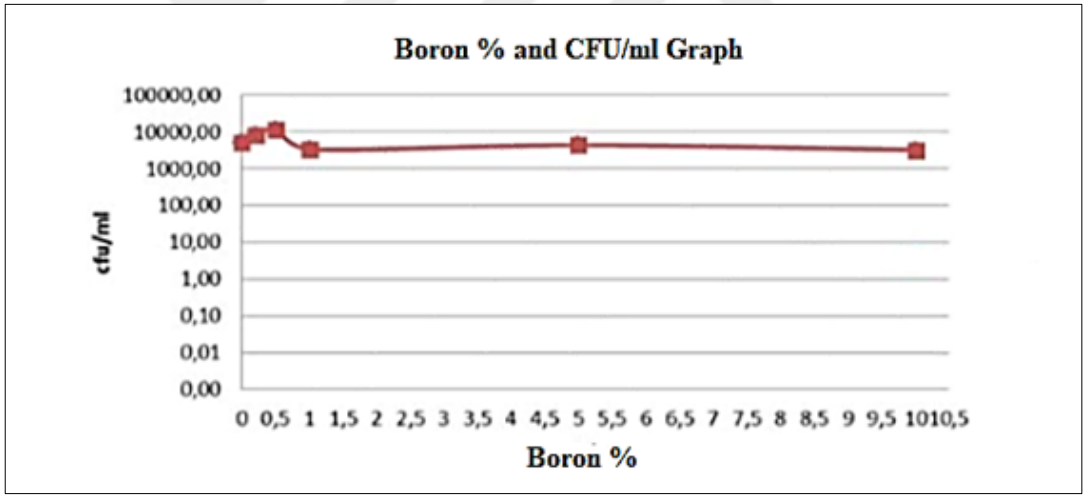


Figure 3.10**.** Number of bacteria depending on boron values

3.7. RESULTS OF FREEZING POINT DETERMINATION

According to the results obtained, the freezing points of Sample 1 was -15 ºC and Sample 2 was -18 ºC. The freezing point of Sample 3 and Sample 4 was -12 ºC. The freezing point of Sample 5 was -8ºC. The microorganism density was mostly in Sample 2, which increased as the boron ratio increased and the size of the microorganisms is also increased (Figure 3.11).

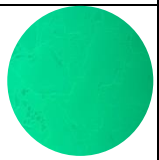
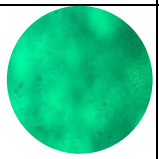
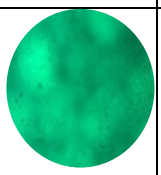
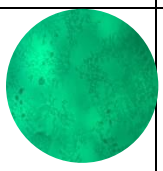
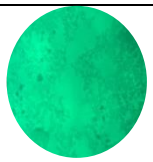
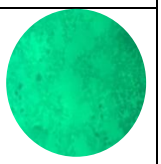
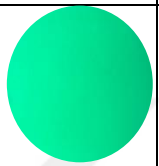
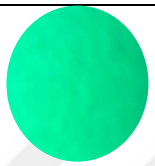
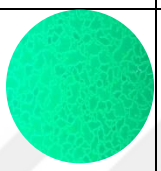
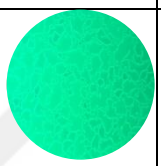
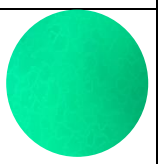
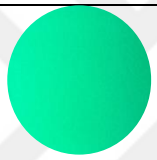
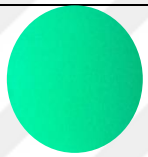
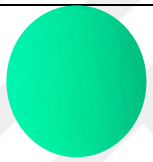
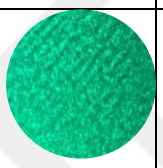
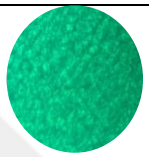
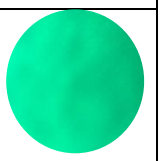
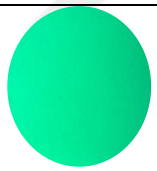
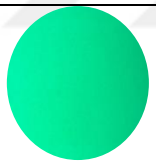
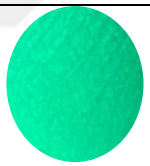
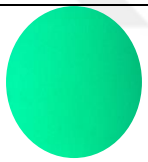
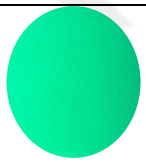
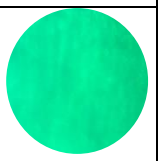
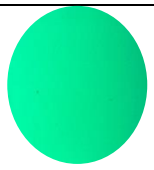
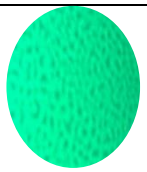
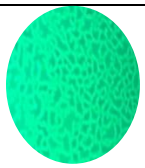
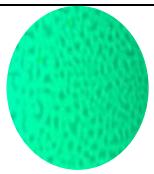
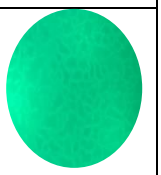
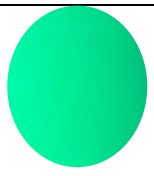
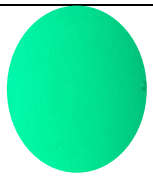
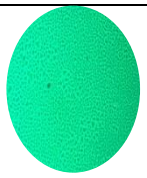
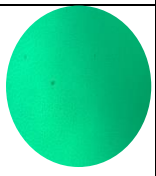
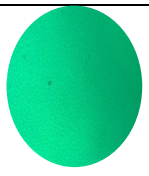
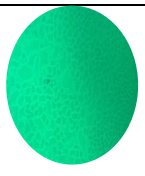
Sample name	0 ºC	-5 ºC	-10 ºC	-15 ºC	20 ºC	-25 ºC
CONTROL						
SAMPLE 1 0.1 percent boron						
SAMPLE 2 0.5 percent boron						
SAMPLE 3 1 percent boron						
SAMPLE 4 5 percent boron						
SAMPLE 5 10 percent boron						

Figure 3.11. Results of freezing point determination

3.8. MIS ANALYSIS RESULTS FOR *PSEUDOMONAS SYRINGAE*

After obtaining extracts for target microorganism, the data and graphs are taken from GS-MS machine (figure3.12). It is understood from the figure that obtained extract are belong to *Pseudomonas syringae* bacterium.

E14B216.53A [10124] Zülal Page 1

Volume: DATA File: E14B216.53A Samp Ctr: 3 ID Number: 10124
Type: Samp Bottle: 2 Method: RTSBA6
Created: 11/21/2014 3:56:56 PM
Sample ID: Zülal

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
0.6919	454417	0.004	----	6.6764		----	< min rt	
0.6994	9.868E+8	0.016	----	6.7332	SOLVENT PEAK	----	< min rt	
0.7923	927	0.011	----	7.4334		----	< min rt	
1.4001	6754	0.009	1.079	11.4508	10:0 3OH	3.09	ECL deviates 0.003	
1.5171	15048	0.008	1.051	12.0002	12:0	6.71	ECL deviates 0.000	Reference -0.002
1.8229	9451	0.008	1.000	13.2027	12:0 2OH	4.01	ECL deviates -0.001	
1.9013	10489	0.008	0.991	13.4836	12:0 3OH	4.41	ECL deviates 0.001	
2.0453	821	0.009	0.975	13.9996	14:0	0.34	ECL deviates 0.000	Reference -0.002
2.3435	1103	0.009	0.951	14.9997	15:0	----	ECL deviates 0.000	
2.6033	90638	0.009	0.937	15.8371	Sum In Feature 3	36.04	ECL deviates -0.003	16:1 w7c/16:1 w6c
2.6536	60165	0.009	0.935	15.9996	16:0	23.87	ECL deviates 0.000	Reference -0.003
2.9076	941	0.010	0.927	16.8130	17:1 w8c	0.37	ECL deviates -0.002	
2.9388	13631	0.009	0.926	16.9129	17:0 cyclo	5.35	ECL deviates -0.002	
2.9664	1766	0.009	0.925	17.0014	17:0	0.69	ECL deviates 0.001	Reference -0.002
3.2290	34511	0.009	0.921	17.8426	Sum In Feature 8	13.48	ECL deviates -0.005	18:1 w7c
3.2779	2240	0.009	0.920	17.9993	18:0	0.87	ECL deviates -0.001	Reference -0.006
3.3051	309	0.009	0.920	18.0885	18:1 w7c 11-methyl	0.12	ECL deviates 0.003	
3.5446	1260	0.010	0.918	18.8742	Sum In Feature 7	0.49	ECL deviates -0.013	19:0 cyclo w10c/19w6
3.5618	427	0.008	0.918	18.9304	19:0 cyclo w8c	0.17	ECL deviates -0.002	
4.2514	560	0.010	----	21.2245		----	> max rt	
----	90638	---	----	----	Summed Feature 3	36.04	16:1 w7c/16:1 w6c	16:1 w6c/16:1 w7c
----	1260	---	----	----	Summed Feature 7	0.49	19:1w7c/19:1 w6c	19:1 w6c/w7c/19cy
----	-----	---	----	----		----	19:0 cyclo w10c/19w6	
----	34511	---	----	----	Summed Feature 8	13.48	18:1 w7c	18:1 w6c

ECL Deviation: 0.004 Reference ECL Shift: 0.003 Number Reference Peaks: 5
Total Response: 248450 Total Named: 248450
Percent Named: 100.00% Total Amount: 236828

Matches:

Library	Sim Index	Entry Name
RTSBA6 6.00	0.542	Pseudomonas-syringae-syringae
	0.485	Pseudomonas-syringae-phaseolicola
	0.364	Pseudomonas-putida-biotype B/vancouverensis
	0.338	Paucimonas-lemoignei
	0.333	Aquaspirillum-autotrophicum
	0.326	Pseudomonas-fluorescens-biotype B

Figure 3.12. MIS analysis results for *Pseudomonas syringae*

3.9. RESULTS OF INVESTIGATION OF THE ANTAGONISTIC EFFECT OF B FERTILIZER FORMULATIONS ON *PSEUDOMONAS SYRINGAE*

The number microorganisms before incubation was $2.52x10^6$. The viable cell numbers achieved after one-day of incubation was as shown in Table 3.4 and Figure 3.13.

Table 3.4. The viable cell numbers achieved after one-day of incubation

	B percent	**gram**	***Pseudomonas Syringae numbers***
Control *Pseudomonas Syringae*	0	0	$9x10^9$
1	0,2	0,007	$1.1x10^9$
2	0,5	0,0143	$7.8x10^9$
3	1	0,0286	$3.1x10^9$
4	5	0,1428	$8x10^7$
5	10	0,2857	$4x10^7$

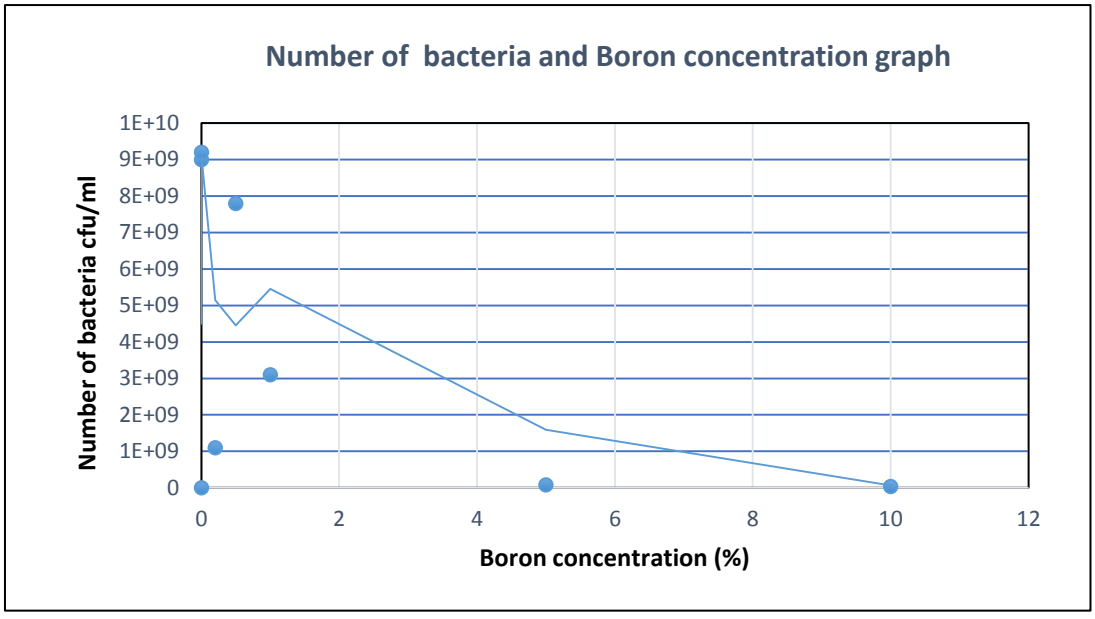


Figure 3.13. The viability graph of *Pseudomonas Syringae*

3.10. THE EFFECTS OF *PSEUDOMONAS SYRINGAE* AND *AZOSPIRILLUM BRASILENSE* ON EACH OTHER

The effect of two microorganisms on each other was investigated by cross – sowing method. No negative effect was detected (Figure 3.14).

Figure 3.14. The effects of *Pseudomonas syringae* and *Azospirillum brasilense* on each other

3.11. CHEMICAL PROPERTIES OF SOILS IN WHICH WHEAT PLANT IS GROWN

Soil samples were taken from 0-30 cm depth and some chemical analyzes were performed. After Bio-B application no statistically significant change in pH values was determined according to the average of 2-year analysis of the soils (p> 0,05) (Table 3.5)

Table 3.5. The pH, organic substance and lime content of the wheat soil

	Application	pH	Organic Substance	Lime
		1:2.5	percentage	
	Control	7,42	1,38	0,76
	Bio-B from soil	7,49	1,44	0,70
1st Year	**Bio-B from foliar**	7,41	1,40	0,75
	Soil+foliar Bio-B	7,44	1,41	0,72
	Control	7,45	1,41	0,81
	Bio-B from soil	7,48	1,46	0,75
2nd Year	**Bio-B from foliar**	7,44	1,45	0,83
	Soil+foliar Bio-B	7,48	1,44	0,71
	Control	7,44a	1,40a	0,79a
	Bio-B from soil	7,49a	1,45a	0,73a
Mean	**Bio-B from foliar**	7,43a	1,43a	0,79a
	Soil+foliar Bio-B	7,45a	1,43a	0,76a

When the changeable cation amounts of the soils were examined, the amount of Ca, K, Mg and P nutrients found in the soil as a result of foliar Bio-B applications were the highest, whereas the changeable Na amount in the soil was obtained from the highest soil by Bio-B application (Table 3.6). These differences were statistically significant ($p < 0.05$). The means marked with different letters (a,b,c,d) in each applications differ significantly ($p > 0.01$) or ($p < 0.05$).

Table 3.6. Soil exchangeable cations (Ca, K, Mg, Na) and P contents

	Application	Ca	K	Mg	Na	P
		me/100 gr				kg/da
	Control	12,12	2,44	2,01	0,24	5,98
	Bio-B from soil	11,23	2,18	1,85	0,32	6,36
1st Year	**Bio-B from foliar**	11,87	2,31	1,98	0,28	6,14
	Soil+foliar Bio-B	11,55	2,27	1,91	0,25	6,22
	Control	12,36	2,30	1,78	0,22	6,25
	Bio-B from soil	11,98	2,24	1,96	0,3	7,25
2nd Year	**Bio-B from foliar**	12,65	2,41	2,15	0,25	7,63
	Soil+foliar Bio-B	12,45	2,36	2,24	0,30	7,41
	Control	12,24a	2,37a	1,90b	0,23c	6,12a
	Bio-B from soil	11,61c	2,21b	1,91b	0,34a	6,81a
Mean	**Bio-B from foliar**	12,26a	2,36a	2,07a	0,27b	6,89a
	Soil+foliar Bio-B	12,00b	2,32a	2,08a	0,28b	6,82a

As a result of the two-year Bio-B fertilizer application, Fe, Cu, Mn and Zn micro element contents of the soil were not statistically significant ($p > 0.05$), while the highest Fe-Bio-B application, Cu-F and Zn were obtained in the control group (Table 3.7). When the B content of the soils was examined, bio-B applications increased the B content of the soils and this increase was statistically significant ($p < 0.01$). The highest soil B content was obtained from the soil by Bio-B application (Figure 3.15) and increased the soil B content by 65 percent compared to the control. These increases were 28 percent with foliar application; 45 percent of soil + foliar application.

Table 3.7. Micro element contents of soils

	Application	Fe	Cu	Mn	Zn	B
		mg/kg				
	Control	2,57	7,12	9,21	4,33	0,41
	Bio-B from soil	2,89	6,78	8,77	4,11	0,62
1st Year	**Bio-B from foliar**	2,64	6,92	9,14	4,25	0,47
	Soil+foliar Bio-B	2,84	6,54	8,95	4,17	0,54
	Control	2,65	4,15	6,52	3,45	0,38
	Bio-B from soil	3,15	4,52	6,77	3,11	0,70
2nd Year	**Bio-B from foliar**	3,21	4,44	5,14	3,36	0,54
	Soil+foliar Bio-B	3,11	4,36	5,45	3,27	0,62
	Control	2,61b	5,64a	7,87a	3,89a	0,40d
	Bio-B from soil	3,02a	5,65a	7,77a	3,61a	0,66a
Mean	**Bio-B from foliar**	2,93a	5,68a	7,14a	3,81a	0,51c
	Soil+foliar Bio-B	2,98a	5,45a	7,20a	3,72a	0,58b

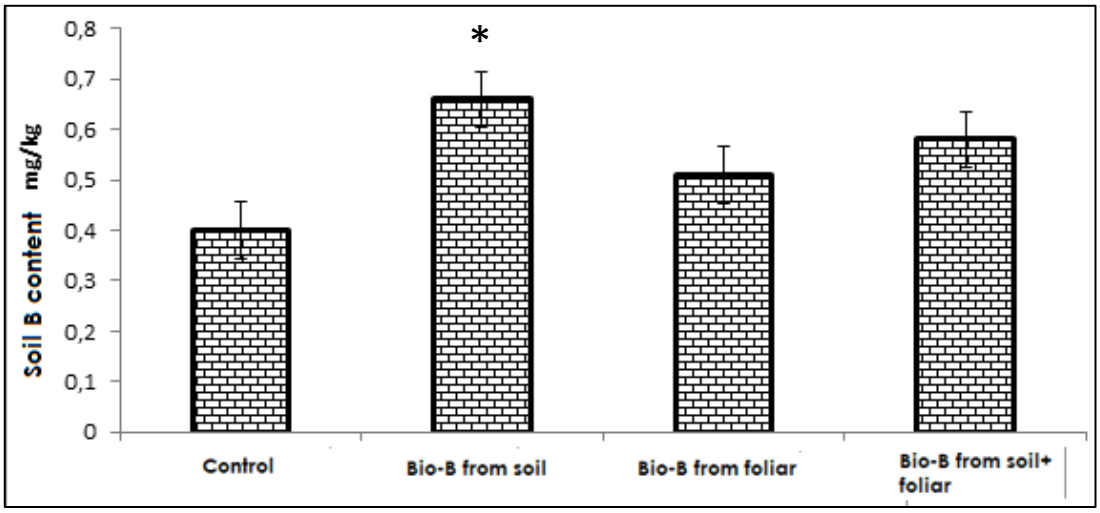


Figure 3.15. The average amount of B in the soil as a result of Bio-B applications

3.12. THE EFFECT OF BIO-B FERTILIZER ON YIELD AND YIELD PARAMETERS OF WHEAT PLANT

Wheat plant samples were taken from the 50x50 cm square frame plots which were established with three replications. For this purpose, the above-ground parts of the harvested wheat plant in square frame thrown into the experimental plot. The measurements were made and converted to decare. Changes in yield of wheat plant due to Bio-B application were determined.

Wheat plants parts were brought to the laboratory and yield and yield parameters of wheat plants were examined. In addition, plant samples from each treatment were dried in air and then allowed to dry in a 68ºC oven for 24 hours. Macro and micro element analyzes were carried out after determination of dry weights of the plant samples which were crushed in a porcelain mortar. For the amino acid, organic acid, hormone, enzyme other biochemical analyzes, samples were taken while the plants were green during spike period and samples were placed in a freezer set to -80 ^{0}C until analysis.

When the effects of Bio-B application on the yield of wheat plant were examined, the results showed that (Table 3.8) yield was statistically significant ($p < 0.01$) and the highest wheat yield was obtained from the soil Bio-B application after two years experiment. Wheat yield increased by 12.88 percent compared to control with Bio-B application from

soil (Figure 3.16). In other applications, wheat yield is increased by 4.68 percent from foliar application and increased by 8 percent from soil+foliar application.

Table 3.8. Yield amounts of wheat plants

	Application	**Yield**
		kg/da
	Control	235,00
	Bio-B from soil	263,44
1st Year	**Bio-B from foliar**	247,56
	Soil+foliar Bio-B	255,47
	Control	242,35
	Bio-B from soil	275,41
2nd Year	**Bio-B from foliar**	252,14
	Soil+foliar Bio-B	260,53
	Control	*238,68d*
	Bio-B from soil	*269,43a*
Mean	**Bio-B from foliar**	*249,85c*
	Soil+foliar Bio-B	258,00b

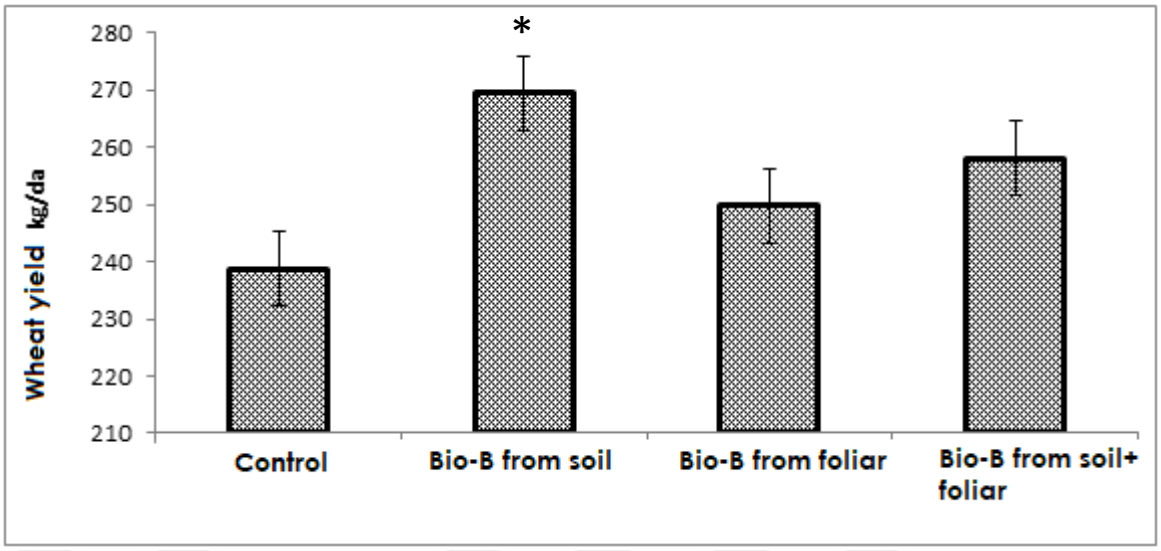


Figure 3.16. Effect of Bio-B application on yield of wheat plant

When the effects of Bio-B applications on the physiological parameters of wheat plant were examined, in terms of two-year average data, chlorophyll, stoma permeability, membrane permeability and photosynthesis were obtained from the highest value with foliar Bio-B application. As a result of the application of Bio-B from the foliar, chlorophyll, stoma permeability, membrane permeability and photosynthesis amount increased by 17.37 percent, 33.33 percent, 5.78 percent and 30.11 percent respectively (Table 3.9).

Table 3.9. Chlorophyll, photosynthesis, stoma and membrane permeability values of wheat plants

	Application	Chlorophyll	Stoma permeability	Membrane permeability	Photosynthesis
		SPAD	mol H_2O $m^{-2}s^{-1}$	percentage	µmol CO_2 $m^{-2}s^{-1}$
	Control	45,00	0,25	57,68	17,38
	Bio-B from soil	48,31	0,27	58,45	18,23
1st Year	**Bio-B from foliar**	51,26	0,31	60,13	24,14
	Soil+foliar Bio-B	47,13	0,30	57,99	20,80
	Control	44,35	0,16	48,45	18,36
	Bio-B from soil	49,15	0,19	49,33	20,11
2nd Year	**Bio-B from foliar**	53,62	0,25	53,14	22,36
	Soil+foliar Bio-B	48,52	0,20	46,52	21,45
	Control	44,68c	0,21d	53,07b	17,87d
	Bio-B from soil	48,73b	0,23c	53,89b	19,17c
Mean	**Bio-B from foliar**	52,44a	0,28a	56,14a	23,25a
	Soil+foliar Bio-B	47,83b	0,25b	52,26b	21,13b

As a result of the analysis of the plant samples taken, the freezing damage rates of the wheat plant at different temperatures were determined and evaluated on the average of two years data. As a result of the evaluation, freeze damage rate decreased due to Bio-B applications and this difference was statistically significant ($p < 0.01$). The highest damage

rate in the control group occurred at -20°C and 98.60 percent (Table 3.10). In case of application of Bio-B to wheat plant at the same temperature, the damage rate was decreased 17.99 percent with soil application; 23.15 percent with foliar application and 21.54 percent with soil + foliar application. Among these values, the most effective application was found to be foliar Bio-B application (Figure 3.17).

Table 3.10. Freezing damage rate of leaves of wheat plant

	Application	0^0C	-5^0C	-10^0C	-15^0C	-20^0C
		percentage				
	Control	46,07	57,53	83,13	95,51	98,52
	Bio-B from soil	27,64	30,12	50,62	58,62	65,36
1st Year	**Bio-B from foliar**	22,11	28,15	47,16	53,26	61,29
	Soil+foliar Bio-B	25,14	29,45	49,63	54,12	62,18
	Control	48,52	58,65	86,53	96,12	98,68
	Bio-B from soil	42,15	53,62	72,41	88,54	96,35
2nd Year	**Bio-B from foliar**	39,65	46,52	65,42	83,52	90,24
	Soil+foliar Bio-B	40,15	47,15	67,41	85,41	92,53
	Control	47,30a	58,09a	84,83a	95,82a	98,60a
	Bio-B from soil	34,90b	41,87b	61,52b	73,58b	80,86b
Mean	**Bio-B from foliar**	30,88d	37,34c	56,29d	68,39c	75,77d
	Soil+foliar Bio-B	32,65c	38,30c	58,52c	69,77c	77,36c

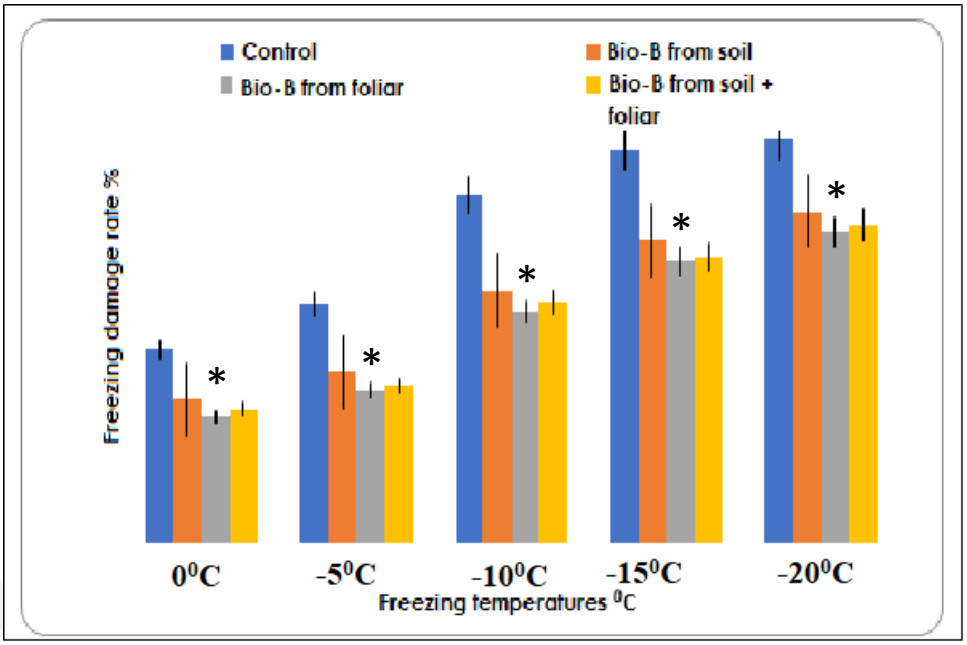


Figure 3.17. Average freezing damage rate of leaves of wheat plant as a result of Bio-B application

When the effects of Bio-B fertilizer applications on the ice nucleation activity of apoplastic protein in the leaves of wheat plant were examined, the highest ice nucleation activity was obtained by application of Bio-B from the leaves and caused an increase of 489 percent compared to the control (Table 3.11).

Table 3.11. Apoplastic protein ice nucleation activity in the leaves of wheat plant

Application		Apoplastic Protein Ice Nucleation Activity
		percentage
	Control	-3,1
	Bio-B from soil	-11,7
1st Year	**Bio-B from foliar**	-18,3
	Soil+foliar Bio-B	-15,4
	Control	-2,7
	Bio-B from soil	-12,3
2nd Year	**Bio-B from foliar**	-15,8
	Soil+foliar Bio-B	-14,6
	Control	-2,9d
	Bio-B from soil	-12,0c
Average	**Bio-B from foliar**	-17,1a
	Soil+foliar Bio-B	-15,0b

When the antioxidant enzyme activity values of wheat plant were examined, it was determined that Bio-B treatment had a significant ($p < 0.01$) effect on CAT, POD and SOD enzyme activity. When the two-year average data were evaluated, the highest CAT and POD enzyme activity was achieved with foliar Bio-B application and the highest SOD enzyme activity was achieved with Bio-B application from soil (Table 3.12).

Table 3.12. Antioxidant enzyme activity of wheat plant

Application		CAT	POD	SOD
		EU gr/ leaf		
	Control	78	1814	42
	Bio-B from soil	85	2012	488
1st Year	**Bio-B from foliar**	93	2231	463
	Soil+foliar Bio-B	82	2102	442
	Control	81	1845	402
	Bio-B from soil	80	2145	512
2nd Year	**Bio-B from foliar**	96	2365	475
	Soil+foliar Bio-B	85	2198	457
	Control	80c	1830c	413c
	Bio-B from soil	83b	2079b	500a
Average	**Bio-B from foliar**	95a	2298a	469b
	Soil+foliar Bio-B	84b	2150b	450b

When the amino acid composition of wheat plant was examined with Bio-B fertilizer application, it was determined that the effect of Bio-B fertilizer application on the amount of amino acid was statistically significant ($p < 0.05$). The highest amounts of aspartate, glutamate, asparagine, serine, glutamine, histidine, glycine, thionine (Table 3.13), arginine, alanine, tyrosine, cystine, valine, methionine, tryptophan, phenylalanine (Table 3.14), isoleucine, leucine, lysine, proline (Table 3.15) amino acid was found Bio-B application from the soil while obtaining the highest hydroxyproline and sarcosine amino acid values with soil + foliar Bio-B application. When the total amount of amino acid was calculated

from these values, the highest total amount of amino acid was obtained from the soil as a result of Bio-B fertilizer application (Figure 3.18).

Table 3.13. The amount of amino acid of wheat plant

	Application	Aspartate	Glutamate	Asparagine	Cerine	Glutamine	Histidine	Glycine	Tionine
		pmol/µl							
	Control	4535	1990	9801	8207	9227	4527	3749	6595
	Bio-B from soil	5412	2522	12152	10526	11524	5462	4715	8366
1st Year	**Bio-B from foliar**	4965	2369	10784	9451	10362	5122	4220	7415
	Soil+foliar Bio-B	5266	2415	11452	9788	10986	5422	4403	7848
	Control	4452	1875	10245	8365	9451	4251	3 52	6451
	Bio-B from soil	5562	2615	12336	11425	12542	5312	4952	8214
2nd Year	**Bio-B from foliar**	5012	2455	11548	9658	11652	5016	4325	7352
	Soil+foliar Bio-B	5389	2568	11658	9654	10245	5324	4652	7754
	Control	4494c	1933b	10023c	8326d	9339c	4389c	3701d	6523d
	Bio-B from soil	5487a	2569a	12244a	10976a	12033a	5387a	4834a	8290a
Mean	**Bio-B from foliar**	4989b	2412a	11166b	9555c	11007b	5069b	4273c	7384c
	Soil+foliar Bio-B	5328a	2492a	11555b	9721b	10616b	5373a	4528b	7801b

Table 3.14. The amount of amino acid of wheat plant

Application		Arginine	Alanine	Tyrosine	Cystine	Valine	Methionine	Tryptophan	Phenyl alanine
		pmol/µl							
	Control	17491	14393	1236	2331	1095	2307	2071	2026
	Bio-B from soil	21635	17659	1569	2856	1352	2715	2458	2415
1st Year	**Bio-B from foliar**	19225	15875	1386	2502	1262	2459	2165	2169
	Soil+foliar Bio-B	20895	16526	1495	2713	1302	2641	2347	2268
	Control	18542	13526	1325	2241	1196	2415	2215	2152
	Bio-B from soil	22154	16528	1699	2745	1469	2865	2311	2451
2nd Year	**Bio-B from foliar**	20163	16528	1415	2659	135	2599	2289	2269
	Soil+foliar Bio-B	21857	17245	1526	2814	1411	2541	2541	2455
	Control	18017c	13960c	1281d	2286d	1146c	2361c	2143b	2089c
	Bio-B from soil	21895a	17094a	1634a	2801a	1411a	2790a	2385a	2433a
Mean	**Bio-B from foliar**	19694b	16202b	1401c	2581c	1307b	2529b	2227b	2219b
	Soil+foliar Bio-B	21376a	16886b	1511b	2764b	1357b	2591b	2444a	2362ab

Table 3.15. The amount of amino acid of wheat plant

	Application	Isolysin	Leucine	Lysin	Hydroxy proline	Sarcosine	Proline
		pmol/µl					
	Control	3078	2731	5133	2073	9726	132
	Bio-B from soil	3784	3451	6255	2415	11859	162
1st Year	**Bio-B from foliar**	3369	3068	5748	2341	0524	142
	Soil+foliar Bio-B	3547	3186	5865	2568	11362	157
	Control	2854	2854	5269	2154	9524	145
	Bio-B from soil	3652	3569	5854	2514	10542	175
2nd Year	**Bio-B from foliar**	3499	3152	5569	2415	11251	157
	Soil+foliar Bio-B	3685	3211	5542	2655	12695	181
	Control	2966c	2793c	5201c	2114c	9625d	139c
	Bio-B from soil	3718a	3510a	6055a	2465b	11201b	169a
Mean	**Bio-B from foliar**	3434b	3110b	5659b	2378b	10888c	150b
	Soil+foliar Bio-B	3616a	3199b	5704b	2612a	12029a	169a

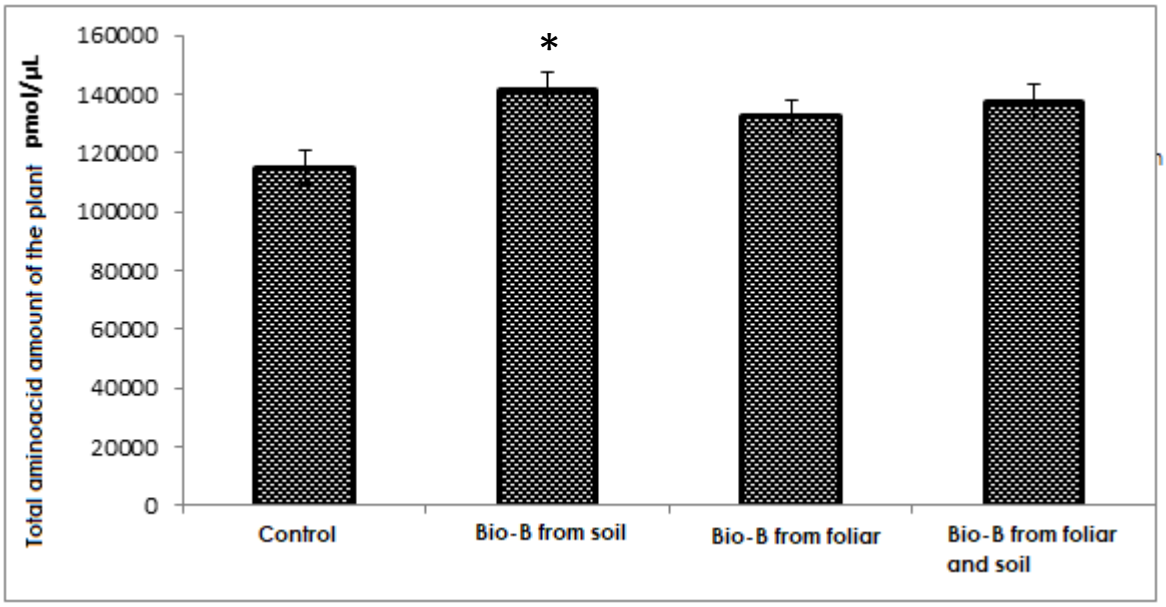


Figure 3.18. Changes in total amino acid amount of wheat plant as a result of Bio-B applications

The amount of organic acid of wheat plant showed significant difference ($p < 0.05$) depending on applied Bio-B fertilizer and the highest oxalic, propionic, tartaric, butyric, malonic, malic, citric, maleic, fumaric and succinic organic acid amounts obtained as a result of soil Bio-B application (Table 3.16). The highest amount of lactic acid was obtained as a result of soil + leaf Bio-B fertilizer application (Table 3.17). When the total organic acid content of the wheat plant was evaluated, the highest amount of organic acid was obtained from the soil as a result of Bio-B fertilizer application and caused an increase of 27.80 percent in the amount of organic acid compared to the control group (Table 3.19).

Table 3.16. Organic acid content of wheat plant

	Application	Oxalic	Propionic	Tartaric	Butyric	Malonic	Malic
		ng/µl					
	Control	1,12	1,75	2,40	3,85	22,31	2,56
	Bio-B from soil	1,36	2,24	2,96	4,62	26,78	3,26
1st Year	**Bio-B from foliar**	1,26	1,90	2,65	4,16	23,69	2,96
	Soil+foliar Bio-B	1,34	2,12	2,85	4,48	25,41	3,10
	Control	1,15	1,84	2,52	3,70	25,42	2,35
	Bio-B from soil	1,42	2,36	3,15	4,52	28,65	3,45
2nd Year	**Bio-B from foliar**	1,36	1,78	2,76	4,26	25,64	3,15
	Soil+foliar Bio-B	1,41	2,24	2,90	4,36	27,84	3,26
	Control	1,14b	1,80b	2,46c	3,78b	23,87b	2,46b
	Bio-B from soil	1,39a	2,30a	3,06a	4,57a	27,72a	3,36a
Mean	**Bio-B from foliar**	1,31a	1,84b	2,71b	4,21a	24,67b	3,06a
	Soil+foliar Bio-B	1,38a	2,18a	2,88b	4,42a	26,63a	3,18a

Table 3.17. Organic acid content of wheat plant

	Application	Lactic	Citric	Maleic	Fumaric	Succinic
		ng/µl				
	Control	18,12	2,55	1,55	0,81	21,35
	Bio-B from soil	22,69	3,26	1,90	1,09	25,55
1st Year	**Bio-B from foliar**	20,63	2,95	1,77	0,87	22,63
	Soil+foliar Bio-B	22,35	3,11	1,80	0,98	24,11
	Control	16,59	2,45	1,36	0,75	20,65
	Bio-B from soil	24,15	3,36	1,85	1,24	26,53
2nd Year	**Bio-B from foliar**	22,13	2,85	1,92	0,95	24,11
	Soil+foliar Bio-B	25,68	3,24	1,76	1,06	25,69
	Control	17,36c	2,50c	1,46c	0,78c	21,00c
	Bio-B from soil	23,42a	3,31a	1,88a	1,17a	26,04a
Mean	**Bio-B from foliar**	21,38b	2,90b	1,85a	0,91b	23,37b
	Soil+foliar Bio-B	24,02a	3,18ab	1,78b	1,02ab	24,90b

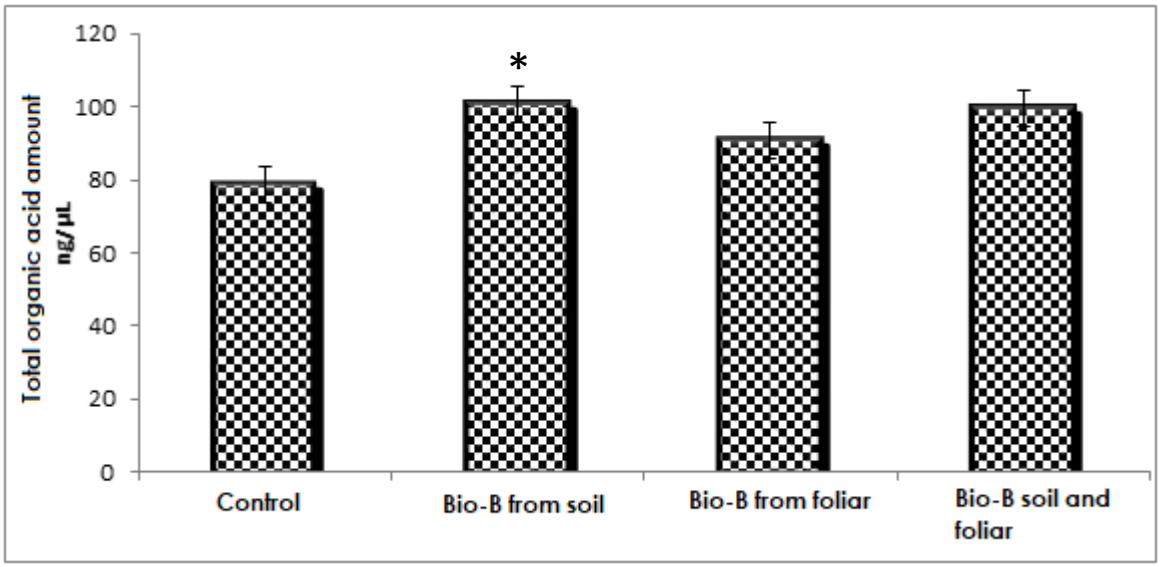


Figure 3.19. Changes in total organic acid content of wheat plant as a result of Bio-B applications

Bio-B fertilizer was applied to wheat plant in different ways, significant changes occurred in the amount of hormone and the level of change was statistically significant ($p < 0.05$). As a result of the two-year study, when the average hormone amounts were evaluated, the highest gibberellic acid and salicylic acid were measured in soil Bio-B application, the highest IAA application were measured in Bio-B foliar application and the highest ABA content were measured in the control group (Table 3.18).

Table 3.18. Hormone amount of wheat plant

	Application	Gibberellic acid	Salicylic acid	IAA	ABA
		ng/µl			
	Control	108,37	35,19	2,48	0,16
	Bio-B from soil	123,24	41,23	2,63	0,15
1st Year	**Bio-B from foliar**	116,57	36,57	2,76	0,14
	Soil+foliar Bio-B	110,94	37,64	2,65	0,15
	Control	115,25	36,52	2,40	0,18
	Bio-B from soil	130,25	44,51	2,70	0,14
2nd Year	**Bio-B from foliar**	124,59	38,54	2,84	0,14
	Soil+foliar Bio-B	116,53	39,12	2,73	0,16
	Control	111,81c	35,86c	2,44b	0,17a
	Bio-B from soil	126,75a	42,87a	2,67ab	0,15b
Mean	**Bio-B from foliar**	120,58b	37,56b	2,80a	0,14b
	Soil+foliar Bio-B	113,74c	38,38b	2,69ab	0,16a

Bio-B fertilizer applied to the wheat plant for two years from soil, foliar and soil + foliar, statistically significant changes occurred in the macro and micro element content of wheat plant, but changes in some nutrients (Ca, Na and Fe) were not found statistically significant. The highest plant N, Ca, K, Na and P content was obtained by soil Bio-B application, while the highest plant Mg content was measured in the control group (Table 3.19).

Table 3.19. Macro nutrient content of wheat plant

	Application	N	Ca	K	Mg	Na	P
		percentage	mg/kg				
	Control	1,54	5232	19834	4213	235	1845
	Bio-B from soil	1,83	5645	21320	3988	275	2231
1st Year	**Bio-B from foliar**	1,61	5342	20143	4045	240	1985
	Soil+foliar Bio-B	1,76	5462	20856	4122	253	2015
	Control	1,45	5462	20154	4415	212	1715
	Bio-B from soil	1,96	5845	22103	4012	241	2154
2nd Year	**Bio-B from foliar**	1,88	5541	21658	4215	202	2211
	Soil+foliar Bio-B	1,92	5695	21748	4369	236	2263
	Control	1,50c	5347a	19994c	4314a	224a	1780b
	Bio-B from soil	1,90a	5745a	21713a	4000c	258a	2193a
Mean	**Bio-B from foliar**	1,75b	5442a	20901b	4130b	221a	2098a
	Soil+foliar Bio-B	1,84a	5579a	21302ab	4246ab	245a	2139a

When the micro element content of wheat plant was evaluated, the highest Fe, Cu and Mn amount was obtained in the control group and the highest Zn amount was obtained by soil + foliar Bio-B application (Table 3.20). Highest B-content of wheat plant was obtained from Bio-B application from foliar. Wheat plant B content was increased 43.84 percent by soil application compared to control; increased 114.96 percent by foliar application; and increased 73.76 percent by soil + foliar application (Figure 3.20).

Table 3.20. Micro-plant nutrient content of wheat plant

	Application	Fe	Cu	Mn	Zn	B
		mg/kg				
	Control	145,36	22,32	40,13	21,3	7,12
	Bio-B from soil	132,13	19,76	32,34	24,31	11,34
1st Year	**Bio-B from foliar**	140,14	21,34	38,76	20,12	17,68
	Soil+foliar Bio-B	136,74	20,13	34,15	28,79	14,37
	Control	136,52	24,15	36,52	18,52	10,25
	Bio-B from soil	140,25	20,16	28,54	22,63	13,66
2nd Year	**Bio-B from foliar**	136,98	24,55	30,10	21,41	19,68
	Soil+foliar Bio-B	141,52	22,98	28,69	23,48	15,82
	Control	140,94a	23,24a	38,33a	19,91c	8,69d
	Bio-B from soil	136,19a	19,96b	30,44c	23,47b	12,50c
Mean	**Bio-B from foliar**	138,56a	22,95a	34,46b	20,77c	18,68a
	Soil+foliar Bio-B	139,13a	21,56ab	31,42c	26,14a	15,10b

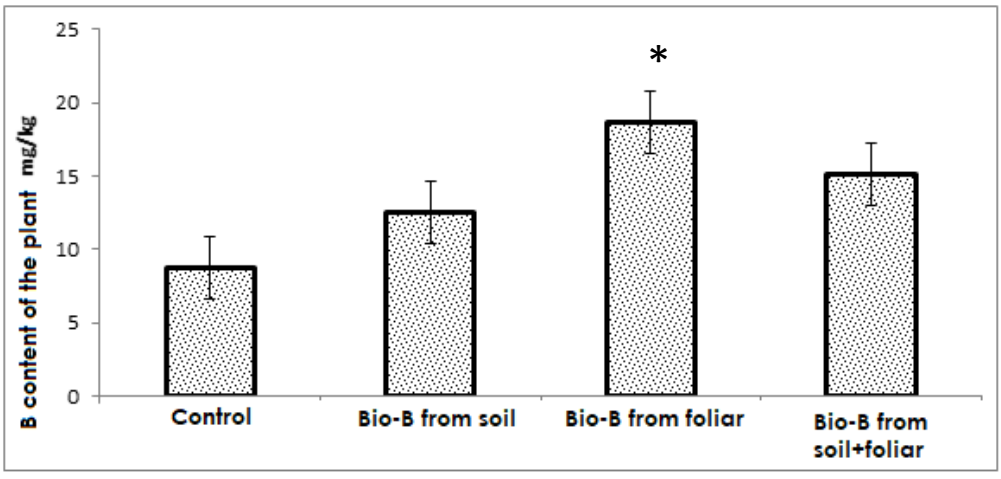


Figure 3.20. Amount of B in the leaves of wheat plant as a result of Bio-B application

The difference of wheat growth between Bio-B fertilizer applied and not applied (control) groups is as in Figure 3.21.

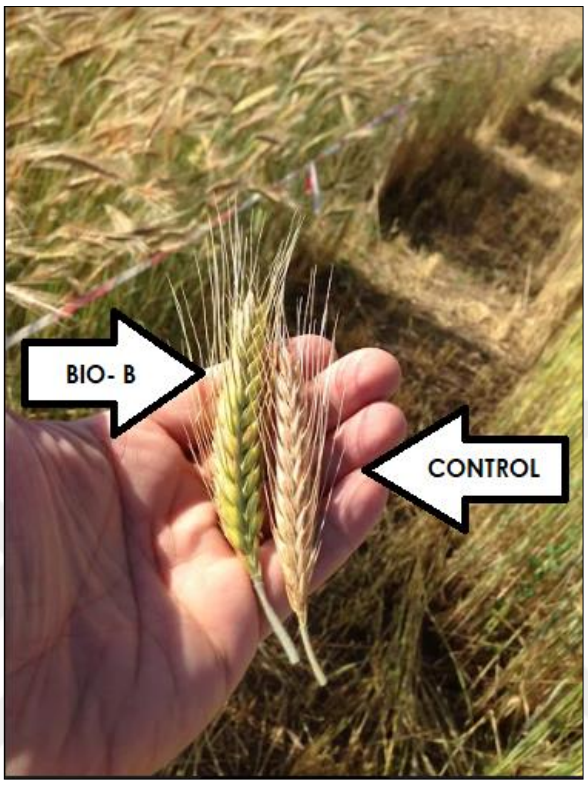


Figure 3.21. The difference of wheat growth between Bio-B fertilizer applied and not applied (control) groups

4. DISCUSSION

The impact of cold stress on especially crop plants is a common research topic. The improvement of cold resistance or chilling tolerance is important for fluidity of the membranes, potential to overcome oxidative stress and final yield in wheat [101]. Bacterial inoculation approaches which include mainly PGPR applications have been found successful to adapt challenging soil and climate conditions for wheat plant. Some PGPRs are also found to be more effective with their influence of phyto-hormone [102].

Turkey's geographic location results with a considerably higher annual variations of temperature changes. Therefore, annual rainfall and especially frost damages cause significant loss of yields in single-year and perennial plants in the region. In each region, significant annual yield and quality losses are reported in single-year and perennial plants depending on the temperature change seen in different periods of the year. Due to this, intense efforts are being made to increase the frost resistance mechanisms of the plants affected by cold stress especially in the recent years. In this study, it was aimed to developed new bio fertilizer formulation under laboratory condition by using boron and PGPR. In addition to this, it was also aimed to determine the antagonistic effects of Bio-B fertilizer formulations applied on *Pseudomonas syringae* which decrease the resistance mechanism against frost damage.

As a result of laboratory tests and biochemical analyzes for the selection of the best fertilizer formulation and most effective and most resistant organism *Azosprillium brasilense* was selected. In addition, efficacy tests of the combined B formulations such as *Pseudomonas syringae* were performed. As a result of this test, the most suitable B level was 0.2 percent and the most suitable Azosprillium brasilense content was 6-7 CFU / ml.

In the field study, Bio-B fertilizer was applied to wheat, in three different ways such as soil, foliar and soil + foliar. As a result of the experiment, it was determined that Bio-B fertilizer had a statistically significant effect on yield and yield parameters. The highest soil B content, the highest wheat yield, the highest amino acid and organic acid content were obtained by using Bio-B from the soil in wheat. The lowest frost damage rate was obtained from the foliar by Bio-B application. Wheat yield increased 12.88 percent compared to the control with Bio-B application in soil. In other applications, wheat yield was 4.68; soil +

foliar application increased by 8 percent. In the case of freezing damage, no Bio-B fertilizer application was applied. 23.15 percent with foliar application and 21.54 percent decrease in soil + foliar application was determined.

In the study of Gunes et al [103] the highest fruit yield was obtained in strawberry plant with soil+ foliar Bio-B application, the lowest frost damage rate and the highest plant B content was obtained from the foliar Bio-B application. According to the control with the application of Bio-B from soil + foliar strawberry yield increased by 55.91 percent. In other applications, the yield of strawberry fruit according to the control has increased 15.88 percent after soil application. However, foliar application caused more increase by 45.28 percent. The freezing damage rate, Bio-B application from the soil in compare with the control (without any Bio-B fertilizer) applied at -200°C decreased by 24.43 percent; 27.41 percent with foliar application and 25.07 percent with soil + foliar application.

In the study of Kose et al [104] the highest fruit grain age and weight was obtained by Bio-B application from soil + foliar, and additionally the lowest frost damage rate; the maximum average bunch weight was obtained by foliar Bio-B fertilizer application. The rate of frost damage in the case of application of Bio-B from the soil according to the control without any Bio-B fertilizer at -200°C was decreased by 21.55 percent with soil application; 25.53 percent with foliar application and 26.24 percent with soil + foliar application.

According to previous studies soil, foliar and soil + foliar combination of Bio-B fertilizer (including *Azospirillum brasilense*) were applied on strawberry cultivation. Both amino acid (23.45 percent) and organic acid (23.45 percent) amounts were increased after Bio-B application [105]. Similarly, in our study the highest B content in soil, the highest wheat yield, the highest amino acid and organic acid content were obtained by using Bio-B from the soil in wheat. Although two studies included different seed types, the results were supporting the benefit of Bio-B fertilizer.

When the literature is evaluated, *Azospirillum* is known as an associative symbiotic PGPB which is predominantly associated with the cereal crops and grasses of the tropics. In a previous study proposed that growth, survival, and activity of the bacterium were highly proposed as temperature-dependent. Kaushik and his colleagues [106] emphasized a low or non-significant effect of *Azospirillum* inoculation in winter crops. Thissituations has

discouraged and decreased the largescale use of this *Azospirillum* bacterium. A similar designed study of the same study group had selected Tn5:lacZ mutants isogenic to wild-type *Azospirillum brasilense* as we used in our study, that were capable of growing at cold temperatures [107]. In field studies, two strains of *Azospirillum brasilense* could influence wheat growth at suboptimal temperatures [106]. Although the temperature regime at which the isolates were assessed for their plant-response, was not precisely temperate, this study constitutes one of the few studies on field performance of *Azospirillum* under suboptimal temperatures and in combination with micronutrient elements. Considering its agronomic significance, *Azospirillum* is a candidate bacterium with the potential for exploration and development of cold-tolerant isolates.

One of the principles of clean and organic agriculture is the reinforcement of the rhizosphere with organic and biological fertilizers. Biological fertilization results vary depending on the type and effectiveness of microorganisms in each BG composition. It is also important that the Bio-B application should not degrade the natural components of the soil over time. In this context, Bio-B application from soil, foliar and soil and foliar did not make a difference in terms of pH, organic matter content and lime component during the 2-year period. The measured values and percentages are similar both in the first- and second-year results and among the groups within themselves.

The literature and the results of our study are consistent with the results of the study and constitute the strength of the study. However, the literature does not include a bio-fertilizer study with both boron and *Azospirillum brasilense*. This aspect of the study has a unique value. For the field studies, tests and productions were carried out to solve the probable problems in large scale production by administrating performance and stabilization studies such as temperature, time, organism viability and load related to the processes of the production of pilot scale. Samples of each production charge were subjected to short and long-term tests and the vitality and efficacy tests of the simulation were completed successfully. The all mentioned process had constituted another strength of this study.

5. CONCLUSION

As a conclusion; Bio-B fertilizer application on wheat plant cultivation is suggested as a beneficial agricultural approach by its significant decreasing effect on freezing damage. In this study, results are suggested that maximum plant nutrition for plant especially boron element diminished the hazard of freeze injury due to the plant ion equilibrium and membrane alterations. Additionally, the application Bio-B was found to be potential to induce anti-oxidant enzyme activity, increase amino acid amount, soil B content and crop yield in wheat plant.

REFERENCES

1. Pandey P, Irulappan V, Bagavathiannan MV. Impact of combined abiotic and biotic stresses on plant growth and avenues for crop improvement by exploiting physio-morphological traits. *Frontiers in Plant Science.* 2017; 8(4):1–15.

2. Pearce RS. Plant Abiotic Stress. *Experimental Agriculture*. 2005;43(02):257.

3. Zhu JK. Abiotic stress signaling and responses in plants. *Cell.* 2016;167(2):313–24.

4. Ait Barka E, Nowak J, Clement C. Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, *Burkholderia phytofirmans* strain PsJN. *Applied and Environmental Microbiology*. 2006;72(11):7246–52.

5. Snyder RL, Melo-Abreu JP de. *Frost protection: fundamentals, practice and economics*. Rome: Food and Agriculture Organization of the United Nations; 2005.

6. Wilkinson RE. *Plant – Environment Interactions*. New York: Marcel Dekker; 2000.

7. Griffith M, Lumb C, Wiseman SB, Wisniewski M, Johnson RW, Marangoni AG. Antifreeze proteins modify the freezing process in planta. *Plant Physiology*. 2005;138(1):330–340.

8. Yamazaki T, Kawamura Y, Uemura M. Extracellular freezing-induced mechanical stress and surface area regulation on the plasma membrane in cold-acclimated plant cells. *Plant Signal Behavior*. 2009;4(3):231–233.

9. Lee RE, Warren GJ, Gusta LV, Gusta LV. *Biological ice nucleation and its applications*. Minnesota: APS Press; 1995.

10. Alberola C, Lichtfouse E, Navarrete M, et al. Agronomy for sustainable development. *Italian Journal of Agronomy*. 2008; 3(3):77–78.

11. Theocharis A, Clément C, Barka EA. Physiological and molecular changes in plants grown at low temperatures. *Planta*. 2012;235(6):1091-105.

12. Sudesh KY. Cold stress tolerance mechanisms in plants. A review. agronomy for sustainable development. *Springer Verlag/EDP Sciences/INRA.* 2010; 30 (3):101-105.

13. Suzuki K, Nagasuga K, Okada M. The chilling injury induced by high root temperature in the leaves of rice seedlings. *Plant Cell Physiology*. 2008;49: 433–442.

14. Solanke AU, Sharma AK. Signal transduction during cold stress in plants. *Physiology Molecular Biology of Plants*. 2008; 14(1-2):69–79.

15. Steponkus PL, Uemura M, Webb MS. A contrast of the cryostability of the plasma membrane of winter rye and spring oat-two species that widely differ in their freezing tolerance and plasma membrane lipid composition. *Advances in Low-Temperature Biology*. 1993;2:211–312.

16. Gill SS, Tuteja N. *Crop Improvement Under Adverse Conditions*. New York: Springer; 2012.

17. Scebba F, Sebastiani L, Vitagliano C. Changes in activity of antioxidative enzymes in wheat (Triticum aestivum) seedlings under cold acclimation. *Phsiologia Plantarum*. 1998;104:(4):747-752.

18. Baek SH, Kwon IS, Park T, Yun SJ, Kim JK, Choi KG. Activities and isozyme profiles of antioxidant enzymes in intercellular compartment of overwintering barley leaves. *Biochemistry and Molecular Biology*. 2000; 33 (5): 385-390.

19. Pearce RS. Molecular analysis of acclimation to cold. *Plant Growth Regulation*. 1999; 29(2):47-76.

20. Smallwood M, Bowles DJ. Plants in a cold climate. *Philosophical Transactions of the Royal Society London Series B.* 2002; 357(1423): 831-847.

21. Vicuna D. *The role of peroxidases in the development of plants and their responses to abiotic stresses.* Doctoral thesis. Dublin Institute of Technology. 2005.

22. Ertürk Y, Güleryüz M. Erzincan koşullarında bazı yerli ve yabancı kayısı çeşitlerinin düşük sıcaklıklara dayanım derecelerinin belirlenmesi. *Tarım Bilimleri Dergisi*. 2007;13(2):128-136.

23. Aslantaş R. *Erzincan şartlarında yetiştirilen bazı badem (Amygdalus communis L.) çeşit/klon ve tiplerinin vejetatif ve generatif gelişme ile çiçek tomurcuklarının dona dayanım derecelerinin belirlenmesi*. Doctoral thesis. Atatürk University, Institute of Science. 1999.

24. Koc I, Yuksel I, Caetano-Anolles G. Metabolite centric reporter pathway and tripartite network analysis of arabidopsis under cold stress. *Frontiers in Bioengineering and Biotechnology*. 2018;6:121-142.

25. Mutlu S, Karadagoglu O, Atıcı O, Nalbantoglu B. Protective role of salicylic acid applied before cold stress on antioxidative system and protein patterns in barley apoplast. *Biologia Plantarum*. 2013;57(3):507-513.

26. Koc İ, Çalışkaner ZO, Filiz E. Application of data analysis in cold stress: a study of Nicotiana benthamiana. *The Journal of Botany*. 2015;39:1021-1032.

27. Reddy PP. *Plant growth promoting rhizobacteria for horticultural crop protection.* India: Springer; 2014.

28. Beneduzi A, Ambrosini A, Passaglia LM. Plant growth-promoting rhizobacteria (PGPR): their potential as antagonists and biocontrol agents. *Genetics and Molecular Biology*. 2012;35(4):1044-1051.

29. Figueiredo MVB, Seldin L, Araujo FF, Mariano RLR. Plant growth promoting rhizobacteria: fundamentals and applications. *Plant growth and health promoting bacteria*. 2010:21-43.

30. Turan M, Kıtır N, Alkaya Ü, Günes A, Tüfenkçi Ş, Yıldırım E, Nikerel E. Making soil more accessible to plants: the case of plant growth promoting rhizobacteria. *Plant growth*. 2016:61-69.

31. Vejan P, Abdullah R, Khadiran T, Ismail S, Nasrulhaq Boyce A. Role of plant growth promoting rhizobacteria in agricultural sustainability. *Molecules*. 2016;21(5):573-590.

32. Lucy M, Reed E, Glick BR, Applications of free livingplant growth-promoting rhizobacteria. *Antonie Van Leeuwenhoek*. 2004;86(1):1-25.

33. Mishra D, Rajvir S, Mishra U, Kumar SS. Role of bio-fertilizer in organic agriculture: a review. *Research Journal of Recent Sciences*. 2013;2(2012):39–41.

34. Choudhary DK, Sharma KP, Gaur RK. Biotechnological perspectives of microbes in agro-ecosystems. *Biotechnology Letters*. 2011;33(10):1905–1910.

35. Franche C, Lindström K, Elmerich C. Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. *Plant and Soil*. 2009;321(1):35–59.

36. Raymond J, Siefert JL, Staples CR. The natural history of nitrogen fixation. *Molecular Biology and Evolution*. 2004;21(3):541–554.

37. Esitken A, Pirlak L, Turan M, Sahin F. Effects of floral and foliar application of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrition of sweet cherry. *Scientia Horticulturae.* 2006;110(4):324-327.

38. Bhattacharyya PN, Jha DK. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiology and Biotechnology.* 2012;28(4):1327–1350.

39. Fukami J, Cerezini P, Hungria M. Azospirillum: benefits that go far beyond biological nitrogen fixation. *AMB Express*. 2018;8(1):73.

40. Bashan Y, De-Bashan LE. Chapter two-how the plant growth-promoting bacterium *Azospirillum* promotes plant growth—a critical assessment. *Advances in agronomy*. 2010; 108(65):77-136.

41. Estrada-De Los Santos P, Bustillos-Cristales R, Caballero-Mellado J. Burkholderia, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. *Applied and Environmental Microbiology.* 2001;67(6):2790-2798.

42. Fuentes-Ramirez LE, Bustillos-Cristales R, Tapia-Hernandez A, Jimenez-Salgado T, Wang ET, Martinez-Romero E, Caballero-Mellado J. Novel nitrogenfixing acetic acid bacteria, Gluconacetobacter johannae sp. nov. and Gluconacetobacter azotocaptans sp. nov., associated with coffee plants. *International Journal of Systematic and Evolutionary Microbiology.* 2001;51(4):1305-1314.

43. Mirza MS, Mehnaz S, Normand P, Prigent-Combaret C, Moenne-Loccoz Y, Bally R. and Malik KA, Molecular characterization and PCR detection o f a nitrogen-fixing Pseudomonas strain promoting rice growth. *Biology and Fertility of Soils.* 2006;43(2):163-170.

44. Backer R, Rokem JS, Ilangumaran G, et al. Plant growth-promoting rhizobacteria: context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. *Frontiers in Plant Science.* 2018;9:1473.

45. Jensen ES, Hauggaard-Nielsen H. How can increased use of biological N2 fixation in agriculture benefit the environment. *Plant and Soil.* 2003;252(1):177-186.

46. Sashidhar B, Podile AR. Mineral phosphate solubilization by rhizosphere bacteria and scope for manipulation of the direct oxidation pathway involving glucose dehydrogenase. *Journal of Applied Microbiology*. 2010;109(1):1-12.

47. Alori ET, Glick BR, Babalola OO. Microbial phosphorus solubilization and its potential for use in sustainable agriculture. *Frontiers in Microbiology*. 2017;8:971-979.

48. Oteino N, Lally RD, Kiwanuka S, et al. Plant growth promotion induced by phosphate solubilizing endophytic *Pseudomonas* isolates. *Frontiers in Microbiology.* 2015;6:745.

49. Ahmed E, Holmström SJ. Siderophores in environmental research: roles and applications. *Microbial Biotechnology*. 2014;7(3):196–208.

50. Radzki W, Gutierrez Mañero FJ, Algar E, Lucas García JA, García-Villaraco A, Ramos Solano B. Bacterial siderophores efficiently provide iron to iron-starved tomato plants in hydroponics culture. *Antonie Van Leeuwenhoek*. 2013;104(3):321–330.

51. Beneduzi A, Ambrosini A, Passaglia LM. Plant growth-promoting rhizobacteria (PGPR): their potential as antagonists and biocontrol agents. *Genetics and Molecular Biology*. 2012;35(4):1044–1051.

52. Çakmakçı R. Stres koşullarında ACC deaminaze üretici bakteriler tarafından bitki gelişiminin teşvik edilmesi. *Journal of the Faculty of Agriculture*. 2009; 40(1):109-125.

53. Singh RP, Shelke GM, Kumar A, Jha PN. Biochemistry and genetics of ACC deaminase: a weapon to "stress ethylene" produced in plants. *Frontiers in Microbiology*. 2015;6:1255.

54. Shaharoona B, Arshad M, Waqas R, Khalid A. Role of ethylene and plant growth-promoting rhizobacteria in stressed crop plants. *Crop stress and its management*. 2012:429-446.

55. Yang J, Kloepper, JW, Ryu CM. Rhizosphere bacteria help plants tolerate abiotic stress. *Trends Plant Science*. 2010;14(1):1-4.

56. Lindow SE, Leveau JHJ. Phyllospheric Microbiology. *Current Opinion in Biotechnology*. 2002;13(3):238–243.

57. Barka AE, Nowk J, Clement C. Enhancement of chilling resistance of inoculated grapevine plantlets with plant growth promoting rhizobacteria Burkholderia phytofermans strain PsJN. *Applied and Enviromental Microbiology*. 2006;72(11):7246–7252.

58. Katiyar V, Goel R. Siderophore mediated plant growth promotion at low temperature by mutant of fluorescent pseudomonad. *Plant Growth Regulation*. 2004;42(3):239–244.

59. Prevost D, Drouin P, Antoun H. The potential use of cold adapted rhizobia to improve nitrogen fixation in legumes cultivated in temperate regions. *Biotechnological application of cold-adapted organisms.* 1999:161–176.

60. Selvakumar G, Joshi P, Nazim S, Mishra PK, Kundu S, Gupta HS. Exiguobacterium acetylicum strain 1P (MTCC 8707) a novel bacterial antagonist from the North Western Indian Himalayas. *World Journal of Microbiology and Biotechnology*. 2009;25(115):131–137.

61. Trivedi P, Sa T. *Pseudomonas corrugata* (NRRL B-30409) mutants increased phosphate solubilization, organic acid production, and plant growth at low temperatures. *Current Microbiology*. 2008; 56(2):140–144.

62. Mishra PK, Bisht SC, Ruwari P, Selvakumar G, Bisht JK. Enhancement of chilling tolerance of inoculated wheat seedlings with cold tolerant plant growth promoting pseudomonads from N.W. Himalayas. *Abstract of First Asian PGPR Conference*; 2009:ANGRAU.

63. Maheshwari DK. *Plant growth and health promoting bacteria.* Berlin: Springer; 2010.

64. Gómez MM, Mercado EC, Pineda EG. Azospirillum is a rhizobacteria with potential use in agriculture. DES *Biological Journal of Agricultural Biological Sciences University of San Nicolás de Hidalgo Michoacán*. 2015;16(1):11-18.

65. Scimat. Azospirillum SEM photography. [cited 2019 19 May]. Available from www.fineartamerica.com/featured/azospirillum-sem-scimat.html.

66. Döbereiner J, Baldoni VLD, Reis VM. Endophytic occurrence of diazotrophic bacteria in non-leguminous crops. *Azospirillum VI and related microorganisms*. 1995:3-14.

67. Steenhoudt O, Vanderleyden J. Azospirillum, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. *FEMS Microbiol Reviews.* 2000;24(4):487-506.

68. Alexandre G. *Azospirillum brasilense*, a beneficial soil bacterium: isolation and cultivation. *Current Protocols in Microbiology* 2017; 47(1):1-10.

69. Brown PH, Bellaloui N, Wimmer MA, Bassil ES, Ruiz J, Hu H, Pfeffer H, Dannel F, and Römheld V. Boron in plant biology. *Plant Biology*. 2002;4:205-223.

70. Morgan JB, Connolly EL. Plant-soil interactions: nutrient uptake. *Nature Education Knowledge*. 2013;4(8):2.

71. Sillanpaa M. Micronutrient assessment at the country level: An international study. *FAO Soils Bulletin* No.63, FAO, Rome, Italy, 1990.

72. Gezgin S, Dursun N, Hamurcu M, Harmankaya M, Önder M, Sade B, Topal A, Soylu S, Akgün N, Yorgancılar M, Ceyhan E, Çiftçi N, Acar B, Gültekin İ, Işık Y, Şeker C and Babaoğlu M. Determination of B contents of soils in Central Anatolian cultivated lands and its relations between soil and water characteristics. *Boron in Plant and Animal Nutrition.* New York: Kluwer Academic Publishers; 2002.

73. Güneş A, Alpaslan M, Çıkılı Y, Özcan H. The effect of zinc on alleviation of boron toxicity in tomato plants. *Turkish Journal of Agriculture and Forestry*. 2008; 24(2000):505-509.

74. Torun AA. Adana ve Osmaniye bölgesinde mısır üretim alanlarında bitkilerin borla beslenme düzeyi ile bor gübrelemesinin dane verimi ve kalitesi üzerine etkisinin belirlenmesi. *BOREN (Ulusal Bor Araştırma Enstitüsü),* Proje No. 193. 2013.

75. Cakmak I, Römheld V. Boron deficiency-induced impairments of cellular functions in plants. *Plant and Soil*. 1997;193(1):71-83.

76. Miwa K, Fujiwara T. Boron transport in plants: co-ordinated regulation of transporters. *Annals of Botany*. 2010;105(7):1103–1108.

77. Koshiba T, Kobayashi M, Matoh T. Boron deficiency: how does the defect in cell wall damage the cells? *Plant Signaling and Behavior*. 2009;4(6):557–558.

78. Cook FC. Boron: its absorption and distribution in plants and its effect on growth. *Journal of Agricultural Research*. 2016;5(19): 877-891.

79. Dordas C, Apostolides GE, Goundra O. Boron application affects seed yield and seed quality of sugar beets. *Journal of Agricultural Science.* 2007;145(4):377-384.

80. Gezgin S, Hamurcu M, Dursun N, Gökmen F. Değişik bor dozları ve uygulama şekillerinin farklı lokasyonlarda yetiştirilen şeker pancarının yaprak bor içeriği, verim ve kalite üzerine etkisi. *Selçuk Tarım ve Gıda Bilimleri Dergisi*. 2007;21(42),25-35.

81. Kratsch HA, Wise RR. The ultrastructure of chilling stress. *Plant, Cell and Environment*. 2000;23(4):337–350.

82. Huang L, Ye Z, Bell RW, Dell B. Boron nutrition and chilling tolerance of warm climate crop species. *Annals of Botany*. 2005;96(5):755–767.

83. Goldbach HE, Wimmer MA, Chaumont F, Matoh T, Volkmann D, Baluška F. Rapid responses of plants to boron deprivation. *Boron in plant and animal nutrition.* 2002;167–180.

84. Thankamani CK, Madan MS, Srinivasan V, Krishnamurthy KS, Kandiannan K. Application of Azospirillum and nutrients on yield, quality parameters and economics of black pepper. *Indian Journal of Horticulture*. 2014;71(2):292-294.

85. McLean EO. Soil pH and lime requirement properties. *Agronomy*. 1982;9:199-223.

86. Nelson R. *Methods of soil analysis.* New York:1982.

87. Nelson DW, Sommer LE. Total carbon, organic carbon and organic matter. *Methods of soil analysis*.1982:595-579.

88. Olsen SR, Sommers LE. Determination of available phosphorus. In methods of soil

analysis. *American Society of Agronomy*. 1982;2:403.

89. Rhoades JD. Cation exchange capacity. In: methods of soil analysis. *Agronomy.* 1982;9:149-157.

90. Lindsay WL, Norvell WA. Development of a DTPA soil test for zinc, iron, manganese, and copper. *Soil Science Society of America Journal*. 1978;42(3):421-428

91. Helrich K. *Official methods of analysis.* Virginia: Association of Official Analytical Chemists (AOAC); 2005.

92. Lutts S, Kinet JM, Bouharmont J. NaCl-induced senescence in leaves of rice (oryza sativa l.) cultivars differing in salinity resistance. *Annals of Botany*. 1996;78(3): 389–398.

93. Griffith M, Ala PI, Yang DSC, Hon WC, Moffat BA. Antifreeze protein produced endogenously in winter rye leaves. *Plant Physiology*. 1992;10(32):593–596.

94. Angelini R, Manes F, Federico R. Spatial and functional correlation between diamine-oxidase and peroxidase activities and their dependence upon de-etiolation and wounding in chick-pea stems. *Planta*. 1990;182:89-96.

95. Heinrikson RL, Meredith SC. Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivatization with phenylisothiocyanate. *Analytical Biochemical*. 1984;136(1),65–74.

96. Horgan, R., & Kramers, M. R. High performance liquid chromatogaphy of cytokinins. *Journal of Chromatography*. 1979;173:263-270.

97. Koshimizo K, Iwamura H. *Cytokinins*. *Chemistry of Plant Hormones*. Florida: CRC Press Inc; 1986.

98. Morris JW, Doumas P, Morris R, Zaer JB. Cytokinins in vegetative and reproductive buds of Pseudotsuga menziesii. *Plant Physiology*. 1990; 9:67-71.

99. Brenner ML. Modern methods for plant growth substance analysis. *Annual Review of Plant Physiology*. 1991;32(66),511-538.

100. Mertens D. Plants preparation of laboratory sample. *Official methods of analysis.* 2005:1–2.

101. Mishra PK, Bisht SC, Ruwari P, Selvakumar G, Joshi GK, Bisht JK, Bhatt JC, Gupta HS. Alleviation of cold stress in inoculated wheat (Triticum aestivum L.) seedlings with psychrotolerant Pseudomonads from NW Himalayas. *Achieves of Microbiology*. 2011;193(7):497-513.

102. Nabti E, Sahnoune M, Ghoul M, Fischer D, Hofmann A, Rothballer M, Schmid M, Hartmann A. Restoration of growth of durum wheat (Triticum durum var.waha) under saline conditions due to inoculation with the rizosphere bacterium Azospirillum brasilense NH and extracts of the marine alga Ulva lactuca. *Journal of Plant Growth Regulation*. 2010;29(1):6–22.

103. Gunes A, Turan M, Kitir N, Tufenkci MS, Cimrin KM, Yildirim E, Ercisli S. Effects of bio-bor fertilizer applications on fruit yield, antioxidant enzyme activity and freeze injury of strawberry. *Erwerbs-Obstbau.* 2016;58(3):177-184.

104. Kose C, Gunes A, Kaya O, Kitir N, Turan M, Sahin F. Freeze injure and antioxidant enzyme activity of grapevine (*Vitis Vinifera*) under bio-boron fertilizer applications. *Enwerbs-Obstbau*. 2018;60(1):3-60.

105. Kitir N, Gunes A, Turan M, Yildirim E, Topcuoglu B, Turker M, Ozlu E, Karaman MR, Firildak. Bio-boron fertilizer applications affect amino acid and organic acid content and physiological properties of strawberry plant. *Erbews-Obstbau* 2018;61(14):129-137.

106. Kaushik R, Saxena AK, Tilak KVBR. Selection and evaluation of *Azospirillum brasilense* strains capable of growing atsub-optimal temperature in rhizocoenosis with wheat. *Folia Microbiologica.* 2001;46(4):327–332.

107. Kaushik R, Saxena AK, Tilak KVBR. Selection ofTn5:lacZmutants isogenic to wild type *Azospirillum brasilense* strains capable of growing at sub-optimal

temperature. *World Journal of Microbiology and Biotechnolgy*. 2000;16(6):567–570.